The anti-diabetic drug metformin regulates voltage-gated sodium channel Na_v1.7 via the ubiquitin-ligase NEDD4-2

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1 **1. Manuscript Title**2 The anti-diabetic drug metformin regulates voltage-gated sodium channel Na_v1.7 via the ubiquitin-ligase
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47 Abstract

48

49 The anti-diabetic drug metformin has been shown to reduce pain hypersensitivity in preclinical
50 models of chronic pain and in neuropathic pain in humans. Multiple intracellular pathways have been
51 described as metformin targets. Among them, metformin is an activator of the adenosine 5'-
52 monophosphate protein kinase (AMPK) that can in turn modulate the activity of the E3 ubiquitin
53 ligase NEDD4-2 and thus posttranslational expression of voltage gated sodium channels (Na_vs). In this
54 study, we found that the bulk of the effect of metformin on Na_v1.7 is dependent on NEDD4-2. In HEK
55 cells, the expression of Na_v1.7 at the membrane fraction, obtained by a biotinylation approach, is
56 only reduced by metformin when co-transfected with NEDD4-2. Similarly, in voltage clamp
57 recordings, metformin significantly reduced Na_v1.7 current density when co-transfected with NEDD4-
58 2. In mouse dorsal root ganglion (DRG) neurons, without changing the biophysical properties of
59 Na_v1.7, metformin significantly decreased Na_v1.7 current densities, but not in *Nedd4L* knockout mice
60 (*SNS-Nedd4L*^{-/-}). In addition, metformin induced a significant reduction in NEDD4-2 phosphorylation
61 at the Serine 328 residue in DRG neurons, an inhibitory phosphorylation site of NEDD4-2. In current
62 clamp recordings, metformin reduced the number of action potentials elicited by DRG neurons from
63 *Nedd4L*^{fl/fl}, with a partial decrease also present in *SNS-Nedd4L*^{-/-} mice, suggesting that metformin can
64 also change neuronal excitability in an NEDD4-2-independent manner. We suggest that NEDD4-2 is a
65 **critical** player for the effect of metformin on the excitability of nociceptive neurons; this action may
66 contribute to the relief of neuropathic pain.

67

68

69 Significance Statement

70

71 Metformin is a multi-target, anti-diabetic drug that has shown therapeutic potential to reduce
72 neuropathic pain. The intracellular mechanisms leading to a reduction in hyperexcitability and pain
73 hypersensitivity remain unclear. We found that metformin acts through the activity of the E3-
74 ubiquitin ligase NEDD4-2 to reduce cell surface expression and currents of voltage gated sodium
75 channels (Na_vs), especially the Na_v1.7 isoform. In current clamp experiments, metformin reduced the
76 DRG neuron firing frequency, with a smaller effect in knockout mice (*SNS-Nedd4L^{-/-}*). On the other
77 hand, NEDD4-2 is indispensable for the metformin effect on the rheobase and the resting membrane
78 potential of DRG neurons. These results suggest that NEDD4-2 activity is a crucial actor, although not
79 exclusive, to reduce the excitability of nociceptive neurons.

80 Introduction

81

82 Metformin is an anti-diabetic drug with pleiotropic effects. Although it has been used for
 83 many decades as a first-line treatment to lower hyperglycemia and glycosylated hemoglobin in type
 84 2 diabetes ([Hundal et al. 2000](#); [Sterne 1957](#)), its primary mechanism of action and effect on pain
 85 were found later. Zhou and colleagues (2001) showed that metformin induces the activation of
 86 adenosine 5'-monophosphate protein kinase (AMPK) in hepatocytes, a phenomenon that in turn
 87 regulates transcription factors and downstream signaling pathways ([Zhou et al. 2001](#)), which was
 88 confirmed in humans ([Musi et al. 2002](#)). Patients with diabetes treated with metformin show less
 89 radiculopathy pain ([Taylor et al. 2013](#)) and musculoskeletal pain ([Carvalho-e-Silva et al. 2021](#)). In
 90 patients with fibromyalgia, metformin treatment significantly improved clinical symptoms such as
 91 pain, fatigue, depression, disturbed sleep, and tender points ([Bullón et al. 2016](#)).

92 One of the first studies indicating a potential use of metformin in experimental neuropathic
 93 pain showed reduction of tactile allodynia-like behavior in mice after spared nerve injury (SNI) and
 94 spinal nerve ligation (SNL) ([Melemedjian et al. 2011](#)). In parallel, there was inhibition of the protein
 95 kinase complex mechanistic target of rapamycin complex 1 (mTORC1) and activation of the
 96 extracellular signal-regulated kinase (ERK) pathways, ultimately leading to dysregulation of
 97 translational control ([Melemedjian et al. 2013](#)). In rats, metformin reversed the chronic constriction
 98 injury (CCI)-induced changes in phosphorylated signal transducer and activator of transcription 3 (p-
 99 STAT3) in spinal dorsal horn (SDH) and activation of microglia and astrocytes in the SDH, as well as
 100 decreased pain-like hypersensitivity ([Ge et al. 2018](#)). Metformin also prevented cisplatin-induced or
 101 spinal cord injury (SCI) mechanical and thermal hypersensitivity ([Mao-Ying et al. 2014](#)) and
 102 attenuated the levels of tumor necrosis alpha (TNF α) and interleukin 1beta (IL-1 β) in the spinal cord
 103 ([Afshari et al. 2018](#)).

104 Metformin acts through a multitude of intracellular signaling pathways in which the master
 105 kinase seems to be AMPK. This kinase is activated by an increase in the cytosolic AMP/adenosine

106 triphosphate (ATP) ratio, playing a crucial role in cellular energy homeostasis. A downstream
 107 ubiquitin protein ligase activated by AMPK is neuronal precursor cell expressed developmentally
 108 downregulated-4 type 2 (NEDD4-2), a ligase that acts on the epithelial sodium channel (ENaC) by
 109 downregulating its currents via ENaC–NEDD4-2-induced internalization ([Bhalla et al. 2006](#)). This
 110 mechanism of action has also been demonstrated for potassium channels such as $K_v1.5$ ([Mia et al.](#)
 111 [2012](#)), $K_v1.7$ ([Andersen et al. 2012](#)), and $K_v7.1$ ([Alzamora et al. 2010](#)). AMPK activation could similarly
 112 enhance NEDD4-2 function and favor sodium channel internalization in sensory neurons. In a
 113 previous study, researchers showed that downregulation of NEDD4-2 is associated with an increase
 114 in total I_{Na} and $Na_v1.7$ currents in the SNI model of neuropathic pain. Restoring normal NEDD4-2
 115 expression after SNI prevented full development of hypersensitivity ([Laedermann et al. 2013](#)). This
 116 leaves open the question as to whether metformin can pharmacologically modulate NEDD4-2
 117 function to decrease sodium current expression, in particular $Na_v1.7$, which in turn might explain its
 118 effect in various experimental models of chronic pain.

119 In this study, we test the hypothesis that metformin inhibits I_{Na} and $Na_v1.7$ currents by
 120 decreasing their expression at the plasma cell membrane via NEDD4-2. In a heterologous system, we
 121 demonstrate that co-transfection of $Na_v1.7$ and NEDD4-2 results in a significant decrease in the
 122 expression of the functional form of this channel coupled with lower current densities upon
 123 metformin treatment. In *Nedd4-2^{fl/fl}* mice, we show that metformin reduces both current densities
 124 and membrane expression of $Na_v1.7$ in comparison with the *SNS-Nedd4L^{-/-}* knockout animals.
 125 Furthermore, the excitability of dorsal root ganglion (DRG) neurons decreases after metformin
 126 treatment, there are altered parameters of single action potentials (APs), and there is
 127 hyperpolarization of the resting membrane potential (RMP). Our results show that metformin
 128 reduces both the expression and current densities of $Na_v1.7$ in a NEDD4-2-dependent manner,
 129 whereas neuronal excitability decreases in both *Nedd4-2^{fl/fl}* and *SNS-Nedd4L^{-/-}* knockout animals.
 130 These findings provide new avenues for research investigating the effect of metformin on neuronal
 131 excitability independently of NEDD4-2.

132 Materials and methods

134 Study approval

135 All experiments involving animals were performed according to the regulations of the
 136 University of Lausanne animal care committee. This investigation conformed to the Guide for the
 137 Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health ([Guide for
 138 the Care and Use of Laboratory Animals 1996](#)), and to the International Association for the Study of
 139 Pain guidelines for the use of animal in research ([Zimmermann 1983](#)).

141 Animals

142 Animals were grouped housed, in a standard environment (litter, paper roll, and tissue), with
 143 free access to food and water, and a 12-h photoperiod. The transgenic mouse line *SNS-Nedd4L*^{-/-} and
 144 their control littermates *Nedd4L*^{fl/fl} were used. Briefly, the floxed *Nedd4L* mouse line (*Nedd4L*^{tm1.1Blyg},
 145 MGI Cat# 3846430, RRID:MGI:3846430, kindly provided by Prof. O. Staub, University of Lausanne,
 146 Switzerland) was crossed with the *SNS-Cre* line (Tg(Scn10a-cre)1Rkun, MGI Cat# 3042874,
 147 RRID:MGI:3042874, kindly provided by R. Kuner, University of Heidelberg, Germany) expressing the
 148 *Cre* recombinase under the promoter of Na_v1.8 (*SNS*). This breeding leads to a conditional knockout
 149 of NEDD4-2 in sensory Na_v1.8-positive DRG neurons. Five-to-eight-week-old animals were used for
 150 experiments. Males and females were used for the experiments.

152 Cell culture and transfection

153 Human embryonic kidney 293 cells (HEK293, ECACC Cat# 12022001, RRID: CVCL_0063) were
 154 cultured in Dulbecco's Modified Eagle's Medium (DMEM, #21885-025, Thermo Fisher Scientific)
 155 supplemented with 10% heat inactivated fetal bovine serum (FBS, #10082, Thermo Fisher Scientific)
 156 and 1% penicillin–streptomycin (P/S, P0781, Sigma-Aldrich), at 37°C in a 5% CO₂ incubator. For patch
 157 clamp experiments, in a T25 culture flask (#353108, Corning Inc.), the cells were seeded at a final
 158 density of 2×10^6 cells and transfected using the Ca²⁺-phosphate method with different conditions: 1
 159 µg of Na_v1.7 complementary DNA (cDNA) concomitantly with 0.3 µg of NEDD4-2 (low NEDD4-2

condition, ratio 1:0.3) + 0.7 μ g of empty vector, 1 μ g of NEDD4-2 (high NEDD4-2 condition, ratio 1:1) alone, or 1 μ g of empty vector alone (no NEDD4-2 condition). In all cases, 0.1 μ g pIRES-AcGFP1 (#632435, Takara Bio Inc.) was added and used as a reporter gene. For biotinylation assays, HEK293 cells were cultured in a P100 dish (#353003, Corning Inc.) at a final density of 9×10^6 cells and transiently co-transfected with 6 μ g of Na_v1.7, as well as 1.8 μ g of NEDD4-2 + 4.2 μ g empty vector (low NEDD4-2 condition), 6 μ g of NEDD4-2 (high Nedd4-2 condition), or 6 μ g of empty vector (no NEDD4-2 condition), mixed with 30 μ L of JetPEI (#101-10N, Polyplus Transfection) and 250 μ L of 150 mM NaCl (#702-50, Polyplus Transfection). The QuickChange mutagenesis kit (#200519, Agilent Technologies, Stratagene) was used to generate NEDD4-2CS mutants by changing Cys801 into a Ser. The cells were used in patch clamp or biochemical experiments 48 h post-transfection.

Primary neuronal culture

Mice were sacrificed with cervical dislocation/lethal injection of pentobarbital (50 mg/kg). For electrophysiological recordings, L4 and L5 DRGs were dissected and collected in oxygenated complete saline solution (CSS composition: 137 mM NaCl, 5.3 mM KCl, MgCl₂, 6H₂O, 25 mM Sorbitol, 10 mM HEPES, 3 mM CaCl₂; pH adjusted to 7.2 with NaOH). DRGs were then harvested and digested in 5 mL of solution containing: Liberase blendzyme TH (#5401151001, Roche) at a concentration of 0.5 U/DRG, 12 μ M ethylenediaminetetraacetic acid (EDTA, #E5134, Sigma-Aldrich) in oxygenated CSS for 20 min at 37°C. Neurons were further digested with Liberase blendzyme TM (#5401127001, Roche) in 5 mL of solution (0.5 U/DRG, 12 μ M EDTA in 5 mL CSS) + 30 U/mL papain (#P3125, Sigma-Aldrich) for 10 min. Then, neurons were suspended in 1 mL of DRG medium containing DMEM/F12 (#21331-020, Thermo Fisher Scientific) with 10% FBS and 1% P/S, supplemented with 1.5 mg of trypsin inhibitor (#T6522, Sigma-Aldrich) and 1.5 mg of purified bovine serum albumin (BSA, #A9647, Sigma-Aldrich). Mechanical dissociation (12 strokes) was performed to triturate the DRGs gently. Finally, isolated neurons were plated on 12-mm coverslips (#631-1577, VWR) coated with 0.1 mg/mL poly-D-lysine (#P7886, Sigma-Aldrich). Neurons were only recorded at 12 h to prevent neurite

186 outgrowth that degrades the space clamp. For cell-surface biotinylation experiments, L2–L6 DRGs
 187 were dissected from each side of the spinal cord and pooled to obtain 10 DRGs per sample. The
 188 dissociated neurons were split in two, for the control and the metformin condition, plated on 6-well
 189 plates (#353224, Corning Inc.), and kept in culture for 6 days before metformin treatment.

190

191 **Cell surface biotinylation assay**

192 HEK293 cells transiently co-transfected or DRG neurons in culture were washed with cold 1X
 193 phosphate-buffered saline pH 7.4 (PBS, #10010, Thermo Fisher Scientific) and then treated with 0.5
 194 mg/mL EZ-link™ Sulfo-NHS-SS-Biotin (#21331, Thermo Fisher Scientific) in cold 1X PBS for 30 min at
 195 4°C. The cells were then washed three times with 200 mM glycine (#A1067, AppliChem) in cold 1X
 196 PBS to inactivate biotin, and twice with cold 1X PBS to remove excess biotin. The cells were scraped
 197 and lysed with 1X lysis buffer that contained 50 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EGTA (pH
 198 8), 10% glycerol, 1% Triton X-100, 10 mM *N*-ethylmaleimide, complete protease inhibitor cocktail
 199 (#11697498001, Roche), and phosphatase inhibitor cocktail (#04906837001, Roche) for 1 h at 4°C on
 200 a wheel. Whole cell lysates were centrifuged at 16,000 *g* for 15 min at 4°C. The protein concentration
 201 of supernatants was measured by using a Bradford-based assay (#500-0006, BioRad). Subsequently, 2
 202 mg (500 µg for neurons) of the supernatant was incubated with 30 µL of Streptavidin Sepharose High
 203 Performance beads (#17-5113-01, GE Healthcare) for 2 h at 4°C on the wheel, and the remaining
 204 supernatants were kept as input fractions (S0 fractions). The beads were subsequently washed five
 205 times with 1X lysis buffer + 1 mM phenylmethanesulfonyl fluoride (#P7626, Sigma-Aldrich) with
 206 centrifugation steps (1000 *g* for 2 min at 4°C) between washes (the supernatant was discarded after
 207 each centrifugation). Elution was done with 40 µL of 5X sample buffer containing 1.5 M sucrose, 10%
 208 sodium dodecyl sulfate (SDS, #L4390, Sigma-Aldrich), 12.5 mM EDTA, 0.3 M Tris (pH 8.8), 0.25%
 209 bromophenol blue, and 150 mM dithiothreitol (#A1101, AppliChem), at 37°C for 1 h with gentle
 210 shaking. A last centrifugation at 2500 *g* for 1 min was performed to separate the beads from the
 211 supernatant fractions. These biotinylated fractions (S2 fractions) were analyzed by western blot for

Na_v1.7 expression at the cell surface. The input fractions were diluted 1:5 in 5X sample buffer, incubated at 37°C for 30 min, and used to evaluate the total expression of Na_v1.7 and the other proteins of interest by western blot.

Western blot

Proteins were separated on 7.5% acrylamide gels (#10681, SERVA Electrophoresis GmbH) by using SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (#162-0177, BioRad). Subsequently, the membranes were immunoblotted with the following antibodies: anti-AMPK (1:1000 in BSA, rabbit polyclonal, Cell Signaling Technology Cat# 2532, RRID:AB_330331), anti-phospho-AMPK (1:1000 in BSA, rabbit polyclonal, Cell Signaling Technology Cat# 2531, RRID:AB_330330), anti-Na_v1.7 (1:500 in milk, mouse monoclonal clone N68/6, Antibodies Incorporated Cat# 75-103, RRID:AB_2184355), anti-NEDD4-2 (1:1000 in milk, rabbit polyclonal, Abcam Cat# ab46521, RRID:AB_2149325), anti-S328 phospho-NEDD4-2 (1:500 in milk, rabbit polyclonal, Abcam Cat# ab95399, RRID:AB_10679598, kindly provided by O. Staub, Lausanne University), and anti- α -tubulin used as a reference protein (1:20,000 in milk, mouse monoclonal clone B-5-1-2, Sigma-Aldrich Cat# T5168, RRID:AB_477579). We used secondary peroxidase-linked goat anti-rabbit or goat anti-mouse IgG (1:10,000, Agilent Cat# P0448, RRID: AB_2617138 and Agilent Cat# P0447, RRID:AB_2617137, respectively) and SuperSignal West Dura Extended Duration Substrate (#34075, Thermo Fisher Scientific) for detection. Chemiluminescence was detected by using the LAS4000 Fuji system (GE Healthcare, ImageQuant, RRID:SCR_014246) coupled with an integrated CCD camera. Protein quantification was performed by using ImageJ software (Fiji, RRID:SCR_002285; [Schindelin et al. 2012](#)). The quantified signals for the proteins of interest were normalized to reference protein signals.

Solutions

Transfected HEK293 cells and DRG neurons in culture were treated with 20 mM metformin (#LKT-M2076-G025, LKT Laboratories) solubilized in their respective culture media, for 12 h, before

239 assessment of electrophysiological recordings or cell-surface biotinylation assays. The concentration
 240 of metformin applied was the optimum for cell survival according to various dose response assays
 241 (data not shown). Control groups were incubated with their respective culture media only.

242 For HEK293 cells, whole cell voltage-clamp recordings were carried out by using an internal
 243 solution containing 60 mM CsCl, 70 mM aspartic acid, 11 mM EGTA, 1 mM MgCl₂, 1 mM CaCl₂, 10
 244 mM HEPES, and 5 mM Na₂-ATP (pH 7.2, adjusted with CsOH) and an external solution containing 130
 245 mM NaCl, 2 mM CaCl₂, 1.2 mM MgCl₂, 5 mM CsCl, 10 mM HEPES, and 5 mM glucose (pH 7.4, adjusted
 246 with CsOH). For DRGs, the internal solution contained 140 mM CsF, 10 mM NaCl, 2 mM MgCl₂, 0.1
 247 mM CaCl₂, 1.1 mM EGTA, and 10 mM HEPES (the pH was adjusted to 7.2 with CsOH and the
 248 osmolarity adjusted to 310 mOsm with glucose). The extracellular solution contained 30 mM NaCl, 20
 249 mM TEA-Cl, 90 mM choline-Cl, 3 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose,
 250 and 0.1 mM CdCl (the pH was adjusted to 7.3 using Tris base and the osmolarity adjusted to 320
 251 mOsm with glucose; [Cummins et al. 2009](#)). The current clamp recordings were carried out by using
 252 an internal solution containing 140 mM KCl, 0.5 mM EGTA, 3 mM Mg-ATP, and 5mM HEPES (pH 7.3,
 253 adjusted with KOH). The extracellular solution contained 140 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 2 mM
 254 MgCl₂, and 10mM HEPES (the pH was adjusted to 7.3 using NaOH and the osmolarity adjusted to 320
 255 mOsm with glucose). See the Extended Data Table 1 for the full list of products.

256

257 **Electrophysiology**

258 Data were acquired with a multiclamp Axon amplifier 700B and pClamp 10 software (pClamp,
 259 RRID:SCR_011323, Molecular Devices) or with an EPC-9 amplifier and Patchmaster software
 260 (Patchmaster, RRID:SCR_000034, HEKA Electronics). Data were analyzed with KaleidaGraph 4.03
 261 (KaleidaGraph, RRID:SCR_014980, Synergy Software Inc.) and GraphPad Prism 7 (RRID:SCR_002798,
 262 GraphPad Software). Low-pass filtering was set to 5.0 kHz. Resistance of the borosilicate pipettes
 263 (#BF150-86-7.5, Sutter Instrument) was 1.5–3 MΩ. After opening, HEK293 cells were kept at -70 mV
 264 for at least 2 min to dialyze and to equilibrate the cell. DRG neurons were kept at -60 mV for 5 min to

265 dialyze the cell and to allow Na_v1.8 voltage dependencies to stabilize and to inactivate Na_v1.9
 266 currents. Cells were then clamped at -80 mV for 2 min before starting the recordings. The I_{Na} current
 267 densities (pA/pF) were obtained by dividing the peak I_{Na} by the cell capacitance.

268 The current clamp recordings were made on cultured primary neurons from *Nedd4L^{fl/fl}* and
 269 *SNS-Nedd4L^{-/-}* animals. Recordings were made following a 500-ms ramp current ranging from 100 to
 270 500 pA, and the APs were counted and represented at each ramp. Small step pulses of 5 ms with 25
 271 pA increments were done, and the first AP triggered was analyzed for different parameters. Peak
 272 amplitude represents the maximum amplitude of the AP measured from the RMP. The maximum rise
 273 time is the time from the start of the stimulation until the maximum AP amplitude was reached. The
 274 baseline is the voltage amplitude starting from the AP threshold until the start of the AP overshoot
 275 and the rheobase is the minimum current needed to elicit an AP.

276 The voltage-dependent steady-state activation (SSA) curves were determined from I/V
 277 curves, where the Na⁺ current was evoked from a holding potential of -100 mV to test pulses of 100
 278 ms ranging from -80 to +40 mV in increments of 5 mV. For DRG neuron recordings, each step was
 279 preceded by a 3-s pre-pulse at -120 mV. The leakage current was subtracted by using the P/4
 280 procedure in Clampex software. The sodium channel conductance was calculated following the
 281 formula $G_{Na} = I_{Na} / (V_m - V_{rev})$, where I_{Na} is the peak current amplitude, V_m is the current potential, and
 282 V_{rev} is the reversal potential for the current determined by a linear fit. The activation curve for each
 283 individual cell was fitted with a Boltzmann equation with the formula $G_{Na} = G_{max} / (1 + \exp [(V_{1/2} - V_m)$
 284 $/ k])$, where G_{max} is the maximum conductance, $V_{1/2}$ is the potential at which half of the sodium
 285 channels are activated, V_m is the current potential, and k is the slope factor.

286 On the other hand, the voltage-dependent steady-state inactivation (SSI) curves were
 287 measured from a holding potential of -100 mV using 500 ms pre-pulses ranging from -130 to +5 mV,
 288 in increments of 5 mV, followed by a test pulse to 0 mV. The inactivation curves were fitted with the
 289 Boltzmann relationship as follows $I_{Na} = I_{max} / (1 + \exp [(V_{1/2} - V_m) / k])$, where I_{max} is the maximum
 290 value for the sodium current, $V_{1/2}$ is the potential at which I_{Na} is half inactivated, V_m is the membrane

291 potential achieved using a pre-pulse step, and k is the slope factor. Five nanomolar Prototoxin II
 292 (ProTxII, #P0033, Sigma-Aldrich) was used to isolate $Na_v1.7$ mediated currents from DRG primary
 293 culture ([Schmalhofer et al. 2008](#)).

294

295 Statistical analyses

296 For statistical analysis, we used Graph Pad Prism and R software. Data are presented as box
 297 plot with whiskers extending from the 10th to 90th percentiles (min to max, including every
 298 individual points), or means $\pm$ standard error of the mean (SEM). Unpaired t-test with Welch's
 299 correction was used to compare the means between two groups, and two-way repeated measures
 300 analysis of variance (ANOVA), followed by a post hoc analysis using a Sidak's multiple comparison
 301 test, was used to compare the effect of metformin treatment in different conditions; $p < 0.05$ was
 302 considered significant. The statistical tests; $t()$, $F()$, and p values; and the effect size calculated with
 303 Cohen's factors are provided in the Extended Data Table 2.

304

305 Results

306

307 *In vitro*, the effect of metformin on the $Na_v1.7$ current density depends on the presence of Nedd4-2

308 We performed whole cell patch-clamp experiments in HEK293 cells co-transfected with
 309 $Na_v1.7$ and different concentrations of NEDD4-2 plasmids. Figure 1A and 1B show, respectively,
 310 representative traces of the $Na_v1.7$ current and the quantification of the maximum $Na_v1.7$ current
 311 density, from a holding potential of -100 mV to test pulses of 100 ms ranging from -80 to +40 mV in
 312 increments of 5 mV, in the different Nedd4-2 concentration conditions (Extended Data Figure 1-2).

313 Treatment with 20 mM metformin for 12 h did not change $Na_v1.7$ peak current in HEK293
 314 cells transfected only with $Na_v1.7$ (No [NEDD4-2], Control vs. Metformin: $p = 0.3907$, $t(51.07) =$
 315 0.8657 , $n = 20$ or 47 /group). When co-transfected with NEDD4-2 at a high concentration ($Na_v1.7$
 316 cDNA/NEDD4-2 cDNA was 1:1 ratio, High [NEDD4-2]), the reduction in $Na_v1.7$ due to the ubiquitin
 317 ligase probably reaches its maximum effect, masking the effect of metformin treatment on $Na_v1.7$

318 peak current (High [NEDD4-2], Control vs. Metformin: $p = 0.2456$, $t(13.2) = 1.215$, $n = 12/\text{group}$). We
319 thus used a lower [NEDD4-2] concentration ($\text{Na}_v1.7$ cDNA/NEDD4-2 cDNA ratio was 1:0.3, termed
320 Low [NEDD4-2]), and observed that the metformin treatment led to a significant decrease in $\text{Na}_v1.7$
321 peak current (Low [NEDD4-2], Control vs. Metformin: $p = 0.0034$, $t(26.57) = 3.221$, $n = 21/\text{group}$).
322 Moreover, in HEK293 cells co-transfected with a mutated NEDD4-2 (NEDD4-2CS), in which the
323 catalytic site is inactivated, the metformin treatment did not change Na_v peak currents (NEDD4-2CS,
324 Control vs. Metformin: $p = 0.743$, $t(24) = 0.3316$, $n = 13/\text{group}$). Metformin did not modify the $V_{1/2}$ of
325 activation of $\text{Na}_v1.7$ in the Low [NEDD4-2] condition (Control vs. Metformin: $p = 0.4506$, $t(21.1) = -$
326 0.77 , $n = 11\text{--}16/\text{group}$). Nevertheless, the slope of $\text{Na}_v1.7$ activation appeared significantly faster
327 upon metformin treatment (Control vs. Metformin: $p = 0.005$, $t(24.3) = -3.08$, $n = 11\text{--}16/\text{group}$)
328 (Table 1).

329 Then, we investigated whether $\text{Na}_v1.7$ cell-surface expression was changed by metformin.
330 Figure 1C shows representative blots of $\text{Na}_v1.7$ in the total input fraction or in the cell surface
331 biotinylation experiment. While the total amount of $\text{Na}_v1.7$ was unchanged (Input fraction, Control
332 vs. Metformin: $p = 0.3871$, $t(17.47) = 0.8871$, $n = 10/\text{group}$), the fully glycosylated form of $\text{Na}_v1.7$
333 (upper band) was significantly decreased in the plasma membrane fraction (Biotinylated fraction,
334 Control vs. Metformin: $p = 0.0104$, $t(14.99) = 2.926$, $n = 10/\text{group}$; Figure 1D). Metformin had no
335 effect on $\text{Na}_v1.7$ expression in either the absence of NEDD4.2 (Control vs. Metformin: $p = 0.068$,
336 $t(8.144) = 2.104$, $n = 6/\text{group}$) or upon high concentration of NEDD4.2 (Control vs. Metformin: $p =$
337 0.5753 , $t(9.524) = 0.5801$, $n = 6/\text{group}$) co-transfected with $\text{Na}_v1.7$ (see Extended Data Figure 1-1).

339 **Metformin downregulates total I_{Na} and $\text{Na}_v1.7$ current densities in DRG neurons via NEDD4-2**

340 We used freshly dissociated L4 and L5 mouse small DRG neurons with a membrane
341 capacitance < 30 pF, considered to be nociceptive neurons ([Lopez-Santiago et al. 2006](#)), and
342 performed whole-cell patch-clamp recordings of total I_{Na} and $\text{Na}_v1.7$ currents (Figure 2A, 2C and
343 Extended Data Figure 2-1). From total I_{Na} currents, we pharmacologically isolated $\text{Na}_v1.7$ current by

344 using 5 nM ProTxII, a concentration at which the toxin blocks Na_v1.7 selectively (Schmalhofer et al.
 345 2008). To investigate the contribution of NEDD4-2 expression in the metformin-mediated
 346 downregulation of the Na_v1.7 current, we used a DRG-neuron-specific NEDD4-2 knockout mouse line
 347 (*SNS-Nedd4L*^{-/-}). Mice heterozygously expressing *Cre* recombinase under the control of the Na_v1.8
 348 promoter (*SNS-Cre*) (Agarwal et al. 2004) were crossed with homozygous mice carrying the *Nedd4-2*
 349 floxed allele (*Nedd4L*^{f/f}) (Shi et al. 2008). In the *Nedd4L*^{f/f} control mice, the total *I*_{Na} was significantly
 350 decreased by the metformin treatment (Total *I*_{Na} *Nedd4L*^{f/f}, Control vs. Metformin: *p* = 0.0139,
 351 *t*(47.72) = 2.554, *n* = 22 or 28/group; Figure 2B, left panel). Conversely, the total *I*_{Na} current density
 352 was not significantly changed in the DRG neurons from the *SNS-Nedd4L*^{-/-} mice (Total *I*_{Na} *SNS-Nedd4L*^{-/-},
 353 Control vs. Metformin: *p* = 0.3637, *t*(48.92) = 0.9169, *n* = 23 or 27 /group; Figure 2B, right panel).
 354 Similarly, a >50% decrease in Na_v1.7 current density was observed upon metformin incubation in the
 355 control mice (Na_v1.7 current density in *Nedd4L*^{f/f}, Control vs. Metformin: *p* = 0.0184, *t*(39.09) =
 356 2.461, *n* = 19 or 23/group; Figure 2D, left panel), but not in the DRG neurons from the *SNS-Nedd4L*^{-/-}
 357 mice (Na_v1.7 current density in *SNS-Nedd4L*^{-/-}, Control vs. Metformin: *p* = 0.4717, *t*(37.18) = 0.7271, *n*
 358 = 20/group; Figure 2D, right panel). Furthermore, similarly to the results in HEK293 cells, metformin
 359 did not modify the *V*_{1/2} of activation of either total *I*_{Na} or Na_v1.7 in DRG neurons from the *Nedd4L*^{f/f}
 360 mice (Table 2).

362 The reduction in currents parallels a downregulation of Na_v1.7 cell-surface expression in DRG 363 neurons

364 Metformin treatment led to a significant decrease in Na_v1.7 expression in the total input
 365 fraction and in the biotinylated fraction of cultured DRG neurons from the control mice (Input
 366 fraction *Nedd4L*^{f/f}, Control vs. Metformin: *p* = 0.0013, *t*(11.79) = 4.205, *n* = 8/group; Biotinylated
 367 fraction *Nedd4L*^{f/f}, Control vs. Metformin: *p* < 0.0001, *t*(13.99) = 5.957, *n* = 8/group; Figure 3B and D,
 368 left panels). By contrast, in the absence of NEDD4-2 in DRG neurons from the *SNS-Nedd4L*^{-/-} mice, no
 369 significant effect of metformin was observed in either the input or biotinylated fraction (Input

fraction *SNS-Nedd4L^{-/-}*, Control vs. Metformin: $p = 0.8917$, $t(7.794) = 0.1407$, $n = 5-6/\text{group}$;
 Biotinylated fraction *SNS-Nedd4L^{-/-}*, Control vs. Metformin: $p = 0.3423$, $t(9.066) = 1.002$, $n = 6/\text{group}$;
 Figure 3B and D, right panels).

Among the kinases activated by metformin that regulate NEDD4.2 function ([Melemedjian et al. 2011](#)), we confirmed here that metformin increased AMPK phosphorylation in DRG neurons (see Extended Data Figure 3-1).

Metformin decreases Nedd4-2 phosphorylation at Ser-328

AMPK-mediated inhibition of ENaC currents has been suggested to be dependent on NEDD4-2 stabilization through phosphorylation at Ser-444 ([Ho et al. 2018](#)). Conversely, NEDD4-2-Ser-328 phosphorylation has been described to prevent the binding of NEDD4-2 on its targets ([Flores et al. 2005](#)). In the current study, metformin did not change NEDD4-2 expression (Total NEDD4-2, Control vs. Metformin: $p = 0.1221$, $t(5.916) = 1.803$, $n = 4/\text{group}$), but significantly decreased NEDD4-2 phospho-Ser328 (p-NEDD4-2, Control vs. Metformin: $p = 0.0117$, $t(4.005) = 4.391$, $n = 4/\text{group}$; Figure 4A and B). Consequently, we found a significant decrease in the p-NEDD4-2/NEDD4-2 ratio when treated with metformin in comparison with regular medium (p-NEDD4-2/NEDD4-2 ratio, Control vs. Metformin: $p = 0.0019$, $t(5.847) = 5.328$, $n = 4/\text{group}$). That change could allow the non-phosphorylated form of NEDD4-2 to bind more efficiently to its substrate (e.g., Na_v1.7), promoting more internalization and reducing I_{Na} and the Na_v1.7 current.

Metformin reduces DRG neuron firing frequencies

A reduction in both total I_{Na} and Na_v1.7 current densities after metformin treatment in the *Nedd4L^{f/f}* mice suggests a reduction in neuronal excitability. We thus recorded DRG neurons in the current clamp configuration (Figure 5A). We found that metformin treatment decreased the number of APs in the *Nedd4L^{f/f}* mice, at increased stimulation ramps (two-way repeated measured ANOVA, Current injection: $F(1.9, 55.8) = 37.49$, $p < 0.0001$; Treatment: $F(1, 28) = 27.3$, $p = 0.0019$; interaction

396 $F(4, 112) = 7.474$, $p < 0.0001$; $n = 14\text{--}16/\text{treatment}$). Post hoc Sidak's multiple comparisons test
 397 revealed a significant treatment effect beginning at 200 pA (200 pA, $p = 0.0413$; 300 pA, $p = 0.0247$;
 398 400 pA, $p = 0.0057$; 500 pA, $p = 0.0021$). There was also a reduction in the number of APs in DRG
 399 neurons from the *SNS-Nedd4L*^{-/-} mice (two-way repeated measured ANOVA, Current injection: $F(1.8,$
 400 $44.5) = 43.16$, $p < 0.0001$; Treatment: $F(1, 125) = 4.6$, $p = 0.0419$; interaction $F(4, 100) = 10.77$, $p <$
 401 0.0001 ; $n = 11\text{--}16/\text{treatment}$) but with less of an impact on neuronal excitability (Post hoc Sidak's
 402 multiple comparisons test, Treatment effect: 200 pA, $p = 0.4772$; 300 pA, $p = 0.2584$; 400 pA, $p =$
 403 0.0399 ; 500 pA, $p = 0.0139$). The resting membrane potential of DRG neurons in the *SNS-Nedd4L*^{-/-}
 404 mice decreased at more negative values after the treatment with metformin (Control vs. Metformin:
 405 $p = 0.0341$, $t(19.18) = 2.282$, $n = 10\text{--}16/\text{group}$), whereas the rheobase significantly increased (Control
 406 vs. Metformin: $p = 0.02$, $t(21.56) = 2.513$, $n = 10\text{--}16/\text{group}$), suggesting metformin affects DRG
 407 excitability independently of NEDD4-2 (Figure 5B). On the other hand, in the *Nedd4L*^{fl/fl} mice the
 408 treatment of cultured DRG neurons with metformin significantly reduced the amplitude (Control vs.
 409 Metformin: $p = 0.0032$, $t(24.04) = 3.278$, $n = 14/\text{group}$), maximum rise time (Control vs. Metformin: $p =$
 410 0.0131 , $t(22.5) = 2.693$, $n = 14/\text{group}$), and baseline of individual APs (Control vs. Metformin: $p =$
 411 0.0043 , $t(25.93) = 3.127$, $n = 14/\text{group}$; Figure 5B). The AP parameters, analyzed from recordings of
 412 *Nedd4L*^{fl/fl} or *SNS-Nedd4L*^{-/-} DRG neurons with or without metformin treatment, are shown in the
 413 Extended Data Figure 5-1. Taken together, our results suggest that mechanisms reducing the
 414 excitability of DRG neurons are partially dependent on NEDD4-2, but also independent of the
 415 ubiquitin ligase.

417 Discussion

418
 419 Despite the recent development of Na_v1.7-specific inhibitors, small molecules, anti-Na_v1.7
 420 antibodies, or other new Na_v isoforms inhibitors ([Alsalam et al. 2020](#)), alternative strategies could
 421 be represented by post-translational regulation of the channel activity for the treatment of chronic
 422 pain ([Laedermann et al. 2015](#)). Metformin has this potential and offers the advantage of being used

423 in clinical settings ([Demaré et al. 2021](#); [Asiedu et al. 2016](#)). We demonstrate here that metformin
 424 induces a decrease in Na_v1.7 channel expression through NEDD4-2 and ultimately reduces the
 425 excitability of the nociceptive neuron.

426 Metformin can enhance the NEDD4-2-mediated epithelial Na⁺ channel ENaC internalization,
 427 in an AMPK-signaling-dependent manner ([Bhalla et al. 2006](#)). AMPK induces ENaC inhibition through
 428 the stabilization of the protein complex including NEDD4-2, thus producing ENaC retrieval from the
 429 plasma membrane, in which the binding motif in the cytoplasmic tail of the β-ENaC subunit is
 430 essential for the interaction with the ubiquitin ligase NEDD4-2 ([Bhalla et al. 2006](#); [Asher et al. 2003](#)).
 431 Similarly, we found increased AMPK phosphorylation with metformin in HEK293 cells, as well as in
 432 cultured DRG neurons, and we observed that NEDD4-2 is essential for the metformin effect on
 433 Na_v1.7 expression at the plasma membrane and Na_v1.7 current density. Ultimately, restoring NEDD4-
 434 2 expression—for example, in experimental neuropathic pain—with a recombinant adeno-associated
 435 viral vector reduced the *I*_{Na} current densities in DRG sensory neurons and repressed the development
 436 of mechanical allodynia following SNI ([Laedermann et al. 2013](#)).

437 Although the mechanisms linking metformin to AMPK phosphorylation have been well
 438 investigated, this drug is a promiscuous compound that affects multiple pathways. For its efficacy,
 439 metformin requires the protein-threonine kinase LKB1, which phosphorylates and activates AMPK
 440 ([Woods et al. 2003](#)). In addition, another upstream kinase of AMPK is Ca²⁺/calmodulin-dependent
 441 protein kinase (CaMKK), which when inhibited pharmacologically or downregulated by using RNA
 442 interference abolished AMPK activation ([Woods et al. 2005](#)). Thus, metformin may act on different
 443 intracellular pathways, independently of AMPK, inducing changes in transcription factors and ion
 444 channel activity. For example, the pleiotropic effect of metformin was investigated in relation to the
 445 phosphorylation of different amino acids, but also with its interaction with the putative kinase
 446 targets such as SGK1 (Serine/threonine-protein kinase), MAP2K2 (dual specificity mitogen-activated
 447 kinase kinase 2), MAPK14 (Mitogen Activated Protein Kinase 14), CDK7 (Cyclin-dependent Kinase 7),
 448 and EGFR (Epidermal Growth Factor Receptor), raising questions related to their downstream chain

of events ([Hart et al. 2016](#)). In our study, we observed a decreased level of NEDD4-2 phosphorylated at Ser-328. This site has been found to be phosphorylated by SGK1 ([Flores et al. 2005](#); [Debonneville et al. 2001](#)). Staub and collaborators found that phosphorylation of NEDD4-2 via SGK1 led to an impairment of ENaC/NEDD4-2 interaction and consequently to more channels at the cell surface ([Flores et al. 2005](#)). Interestingly, Bhalla and colleagues showed an AMPK-dependent modulation of NEDD4-2 controlling ENaC activity, however, they found that the SGK1 pathway was not involved in the AMPK regulation of ENaC ([Bhalla et al. 2006](#)). Because the levels of phosphorylated Ser-328 were altered, our results suggest that the SGK-1 pathway could be involved in this effect. It would be an interesting pathway to explore in parallel to AMPK signaling, especially because the sites of NEDD4-2 phosphorylated by AMPK are not well identified ([Arévalo 2015](#)).

In cultured sensory trigeminal (TG) neurons, metformin induces an increase in AMPK activation and the inhibition of the mTOR pathway ([Melemedjian et al. 2011](#)). Metformin, as well as AMPK activators, can abrogate the phosphorylation of mTOR, 4EBP, and rS6 (for which phosphorylation is increased after nerve injury). The authors also suggested that pharmacological AMPK activation (including by using metformin) negatively normalizes aberrant translational control after peripheral nerve injury, a phenomenon that contributes to reduced pain hypersensitivity in experimental models of neuropathic pain (SNI and SNL).

Metformin could act transcriptionally and post-translationally to decrease neuronal excitability. Interestingly, AMPK activators could potentially have similar mechanisms, as for the allosteric modulator A769662: Acute application of this compound blocked in a dose-dependent manner voltage-gated sodium channels in rat TG neurons primary cultures ([Asiedu et al. 2017](#)). The effect of metformin via NEDD4-2 could also occur through modification of protein expression. We found that the amount of Na_v1.7 in whole-cell lysates of cultured DRG neurons was decreased after metformin treatment, and this regulation was dependent on NEDD4-2. Thus, NEDD4-2 acts on other regulatory machinery of membrane protein internalization, and this could happen directly through an enzymatic post-translational ubiquitination or indirectly. Interestingly, Grimsey and collaborators

(2015) described an atypical role for NEDD4-2 E3 ubiquitin ligase. They showed that different G protein-coupled receptors could stimulate p38 mitogen-activated protein kinase (p38-MAPK) activation via NEDD4-2-mediated ubiquitination ([Grimsey et al. 2015](#)). Thus, NEDD4-2 may also regulate ion channel protein expression via p38-MAPK signaling. Consequently, it was shown that metformin regulates the remodeling of the small conductance calcium-activated potassium channels (SK2 and SK3) in the atrial tissues of a rat model of type 2 diabetes mellitus through the inhibition of NOX4 expression (constitutive NADPH oxidase), but also by significantly suppressing the p38MAPK signaling pathway ([Liu et al. 2018](#)).

Regulation of I_{Na} was also investigated in relation with metformin treatment and interactions with cytoskeleton binding proteins. In human breast cancer MCF-7 cell line metformin decreases phosphorylation of cofilin, an actin-binding protein involved in cytoskeleton dynamics, which is upstream regulated by LIM-kinase and Rho-kinase, suggesting a possible interaction of metformin with Rho-kinase ([Özdemir and Ark 2021](#)). Furthermore, in chick DRG neurons Rho-kinase phosphorylates the collapsin response mediator protein 2 (CRMP2), and inactivates the ability of CRMP2 to promote microtubule assembly and Numb-mediated endocytosis during growth cone collapse ([Arimura et al. 2005](#)). Beside the complex with Numb, CRMP2 interacts also with Eps15 and NEDD4-2 to promote the endocytosis of $Na_v1.7$ as a specific silencing of these targets changed the sodium currents ([Gomez et al. 2021](#); [Dustrude et al. 2016](#); [Cai et al. 2021](#)). But this appears only in relation with the CRMP2's post-translational modification state of SUMOylation. Therefore, we do not exclude this pathway by which metformin, mediated by Rho-kinase and CRMP2, may interact with NEDD4-2 to promote $Na_v1.7$ internalization and loss of sodium currents.

AMPK acts on NEDD4-2 and consequently on the ion channels expressing the intracellular binding motif. This fact confirms our data on sodium current densities in both the heterologous and homologous primary systems in which treatment with metformin decreased the total I_{Na} and $Na_v1.7$ currents with only a marginal effect on the biophysical properties of the channel. The modulation by metformin determined by NEDD4-2, however, is not only restricted at sodium channels. Treatment

501 of mouse polarized kidney cortical cells with metformin inhibits $K_v7.1$ activity by promoting NEDD4-2-
 502 dependent channel ubiquitination ([Alzamora et al. 2010](#)). Interestingly, the expression and current
 503 levels of $K_v1.4$ are downregulated in an AMPK-PKC manner independently of NEDD4-2, or the related
 504 ubiquitin ligase NEDD4-1, and independently of Phosphoinositide 3-kinase PI3K-SGK1 signaling
 505 pathway, suggesting that NEDD4-2 probably exerts different functions depending on the binding
 506 motif of the cytoplasmic tail of ion channels ([Andersen et al. 2018](#)).

507 Our current clamp data is in line with previously published studies indicating that metformin
 508 can reduce neuronal excitability, lowering the frequency of APs ([Melemedjian et al. 2011](#)). $Na_v1.7$ is
 509 one of the most important members of the fast kinetics tetrodotoxin-sensitive sodium channels that
 510 contributes to the rising phase of APs ([Bennett et al. 2019](#)). Therefore, metformin treatment,
 511 lowering $Na_v1.7$ expression, reduces the amplitude and maximum rise time of single APs, whereas
 512 the baseline is higher. We have shown that metformin is also able to partially reduce neuronal
 513 excitability in NEDD4-2 knockout animals, alongside changes in the rheobase and RMP, indicating a
 514 possible effect through different pathways and channels.

515 Finally, a recent study showed that the effect of metformin was not limited to peripheral
 516 neurons, but metformin treatment also altered spinal microglia and astrocytes activation in male
 517 mice after SNI ([Inyang et al. 2019](#)). The authors described several mechanisms of action of metformin
 518 on the androgen-dependent regulation of OCT2 (Organic Cation Transporter-2), but the possibility
 519 that metformin could also regulate microglial ion channels should not be excluded. Indeed, different
 520 potassium currents are increased in activated microglia (e.g., Kir2.1-mediated currents) ([Gattlen et al.](#)
 521 [2020](#); [Madry et al. 2018](#)), which are also regulated by NEDD4-2, and could be the target of
 522 metformin.

523 Our study was not conceived to answer the question of a gender effect during metformin treatment,
 524 but the issue is of particular interest. The link between the cytoskeleton binding protein CRMP2
 525 SUMOylation and $Na_v1.7$ showed a reduction of expression and currents, in female mice compared
 526 with males ([Moutal et al. 2020](#)). Moreover, knocking-down the expression of NEDD4-2 with silencing

RNA in DRG sensory neurons restored the loss of sodium currents, in CRMP2 SUMO-null knock-in (CRMP2K374A/K374A) female mice ([Gomez et al. 2021](#)). Apart the link between CRMP2, NEDD4-2 and Na_v1.7 that is gender different, the treatment with metformin reverses SNI-induced mechanical and cold hypersensitivity in male but not in female mice. This effect might be due, at least in part, to how metformin differently activated microglial cells between the two genders whereas AMPK activity increases in DRG neurons both in male and female ([Inyang et al. 2019](#)). Whether changes into AMPK activity, NEDD4-2 and Na_v1.7 currents after metformin is gender dependent need to be further investigated.

In conclusion, we have provided novel data regarding the mechanism of action of metformin on sodium channels, which play a crucial role in physiological and pathological pain. Comprehension of those mechanisms opens new alternatives for the diminution of hyperexcitability related to neuropathic pain, in particular by targeting post-translational regulation of sodium channels.

References

- Afshari, Khashayar, Amir Dehdashtian, Nazgol Sadat Haddadi, Arvin Haj-Mirzaian, Arad Iranmehr, Mohammad Ali Ebrahimi, Seyed Mohammad Tavangar, Hedyeh Faghir-Ghanesefat, Fatemeh Mohammadi, Nastaran Rahimi, Abbas Norouzi Javidan, and Ahmad Reza Dehpour. 2018. 'Anti-inflammatory effects of Metformin improve the neuropathic pain and locomotor activity in spinal cord injured rats: introduction of an alternative therapy', *Spinal Cord*, 56: 1032-41.
- Agarwal, N., S. Offermanns, and R. Kuner. 2004. 'Conditional gene deletion in primary nociceptive neurons of trigeminal ganglia and dorsal root ganglia', *Genesis*, 38: 122-9.
- Alsaloum, Matthew, Grant P. Higerd, Philip R. Effraim, and Stephen G. Waxman. 2020. 'Status of peripheral sodium channel blockers for non-addictive pain treatment', *Nature Reviews Neurology*, 16: 689-705.
- Alzamora, Rodrigo, Fan Gong, Christine Rondanino, Jeffrey K. Lee, Christy Smolak, Núria M. Pastor-Soler, and Kenneth R. Hallows. 2010. 'AMP-activated protein kinase inhibits KCNQ1 channels through regulation of the ubiquitin ligase Nedd4-2 in renal epithelial cells', *American Journal of Physiology - Renal Physiology*, 299.
- Andersen, Martin N., Katarzyna Krzystanek, Thomas Jespersen, Søren Peter Olesen, and Hanne B. Rasmussen. 2012. 'AMP-activated protein kinase downregulates Kv7.1 cell surface expression', *Traffic*, 13: 143-56.
- Andersen, Martin Nybo, Lasse Skibsbye, Arnela Saljic, Martin Zahle Larsen, Hanne Borger Rasmussen, and Thomas Jespersen. 2018. 'Regulation of Kv1.4 potassium channels by PKC and AMPK kinases', *Channels*, 12: 34-44.

- 563 Arévalo, Juan C. 2015. 'Nedd4-2 regulation of voltage-gated ion channels: an update on structure-
564 function relationships and the pathophysiological consequences of dysfunction', *Journal of*
565 *Receptor, Ligand and Channel Research*: 53.
- 566 Arimura, Nariko, CéLine MéNager, Yoji Kawano, Takeshi Yoshimura, Saeko Kawabata, Atsushi Hattori,
567 Yuko Fukata, Mutsuki Amano, Yoshio Goshima, Masaki Inagaki, Nobuhiro Morone, Jiro
568 Usukura, and Kozo Kaibuchi. 2005. 'Phosphorylation by Rho Kinase Regulates CRMP-2 Activity
569 in Growth Cones', *Molecular and Cellular Biology*, 25: 9973-84.
- 570 Asher, C., I. Sinha, and H. Garty. 2003. 'Characterization of the interactions between Nedd4-2, ENaC,
571 and sgk-1 using surface plasmon resonance', *Biochim Biophys Acta*, 1612: 59-64.
- 572 Asiedu, M. N., G. Dussor, and T. J. Price. 2016. 'Targeting AMPK for the Alleviation of Pathological
573 Pain', *Exp Suppl*, 107: 257-85.
- 574 Asiedu, M. N., C. Han, S. D. Dib-Hajj, S. G. Waxman, T. J. Price, and G. Dussor. 2017. 'The AMPK
575 Activator A769662 Blocks Voltage-Gated Sodium Channels: Discovery of a Novel
576 Pharmacophore with Potential Utility for Analgesic Development', *PLoS One*, 12: e0169882.
- 577 Bennett, D. L., A. J. Clark, J. Huang, S. G. Waxman, and S. D. Dib-Hajj. 2019. 'The Role of Voltage-
578 Gated Sodium Channels in Pain Signaling', *Physiol Rev*, 99: 1079-151.
- 579 Bhalla, Vivek, Nicholas M. Oyster, Adam C. Fitch, Marjolein A. Wijngaarden, Dietbert Neumann, Uwe
580 Schlattner, David Pearce, and Kenneth R. Hallows. 2006. 'AMP-activated kinase inhibits the
581 epithelial Na⁺ channel through functional regulation of the ubiquitin ligase Nedd4-2', *Journal*
582 *of Biological Chemistry*, 281: 26159-69.
- 583 Bullón, Pedro, Elisabet Alcocer-Gómez, Angel M. Carrión, Fabiola Marín-Aguilar, Juan Garrido-
584 Maraver, Lourdes Román-Malo, Jesus Ruiz-Cabello, Ognjen Culic, Bernhard Ryffel, Lionel
585 Apetoh, François Ghiringhelli, Maurizio Battino, José Antonio Sánchez-Alcazar, and Mario D.
586 Cordero. 2016. 'AMPK Phosphorylation Modulates Pain by Activation of NLRP3
587 Inflammasome', *Antioxidants and Redox Signaling*, 24: 157-70.
- 588 Cai, S., A. Moutal, J. Yu, L. A. Chew, J. Isensee, R. Chawla, K. Gomez, S. Luo, Y. Zhou, A. Chefdeville, C.
589 Madura, S. Perez-Miller, S. S. Bellampalli, A. Dorame, D. D. Scott, L. Francois-Moutal, Z. Shan,
590 T. Woodward, V. Gokhale, A. G. Hohmann, T. W. Vanderah, M. Patek, M. Khanna, T. Hucho,
591 and R. Khanna. 2021. 'Selective targeting of NaV1.7 via inhibition of the CRMP2-Ubc9
592 interaction reduces pain in rodents', *Sci Transl Med*, 13: eabh1314.
- 593 Carvalho-e-Silva, Ana Paula, Alison R. Harmer, Manuela L. Ferreira, and Paulo H. Ferreira. 2021. 'The
594 effect of the anti-diabetic drug metformin on musculoskeletal pain: A cross-sectional study
595 with 21,889 individuals from the UK biobank', *European Journal of Pain (United Kingdom)*: 1-
596 10.
- 597 Cummins, T. R., A. M. Rush, M. Estacion, S. D. Dib-Hajj, and S. G. Waxman. 2009. 'Voltage-clamp and
598 current-clamp recordings from mammalian DRG neurons', *Nat Protoc*, 4: 1103-12.
- 599 Debonneville, C., S. Y. Flores, E. Kamynina, P. J. Plant, C. Tauxe, M. A. Thomas, C. Munster, A. Chraibi,
600 J. H. Pratt, J. D. Horisberger, D. Pearce, J. Loffing, and O. Staub. 2001. 'Phosphorylation of
601 Nedd4-2 by Sgk1 regulates epithelial Na⁺ channel cell surface expression', *EMBO J*, 20:
602 7052-9.
- 603 Demaré, Sarah, Asha Kothari, Nigel A. Calcutt, and Paul Fernyhough. 2021. 'Metformin as a potential
604 therapeutic for neurological disease: mobilizing AMPK to repair the nervous system', *Expert*
605 *Review of Neurotherapeutics*, 21: 45-63.
- 606 Dustrude, Erik T., Aubin Moutal, Xiaofang Yang, Yuying Wang, May Khanna, and Rajesh Khanna. 2016.
607 'Hierarchical CRMP2 posttranslational modifications control NaV1.7 function', *Proceedings of*
608 *the National Academy of Sciences*, 113: E8443-E52.
- 609 Flores, Sandra Y., Dominique Loffing-Cueni, Elena Kamynina, Dorothée Daidié, Carole Gerbex, Sting
610 Chabanel, Jean Dudler, Johannes Loffing, and Olivier Staub. 2005. 'Aldosterone-Induced
611 Serum and Glucocorticoid-Induced Kinase 1 Expression Is Accompanied by Nedd4-2
612 Phosphorylation and Increased Na⁺ Transport in Cortical Collecting Duct Cells', *Journal of the*
613 *American Society of Nephrology*, 16: 2279-87.

- 614 Gattlen, Christophe, Alexandru-Florian Deftu, Raquel Tonello, Yuejuan Ling, Temugin Berta, Violeta
615 Ristoiu, and Marc René Suter. 2020. 'The inhibition of Kir2.1 potassium channels depolarizes
616 spinal microglial cells, reduces their proliferation, and attenuates neuropathic pain', *Glia*, 68:
617 2119-35.
- 618 Ge, Anqi, Shu Wang, Bei Miao, and Ming Yan. 2018. 'Effects of metformin on the expression of AMPK
619 and STAT3 in the spinal dorsal horn of rats with neuropathic pain', *Mol Med Rep*.
- 620 Gomez, Kimberly, Dongzhi Ran, Cynthia L. Madura, Aubin Moutal, and Rajesh Khanna. 2021. 'Non-
621 SUMOylated CRMP2 decreases Nav1.7 currents via the endocytic proteins Numb, Nedd4-2
622 and Eps15', *Molecular Brain*, 14.
- 623 Grimsey, Neil J., Berenice Aguilar, Thomas H. Smith, Phillip Le, Amanda L. Soohoo, Manojkumar A.
624 Puthenveedu, Victor Nizet, and Jo Ann Trejo. 2015. 'Ubiquitin plays an atypical role in GPCR-
625 induced p38 MAP kinase activation on endosomes', *Journal of Cell Biology*, 210: 1117-31.
- 626 *Guide for the Care and Use of Laboratory Animals*. 1996. (National Academies Press).
- 627 Hart, Thomas, Shihab Dider, Weiwei Han, Hua Xu, Zhongming Zhao, and Lei Xie. 2016. 'Toward
628 Repurposing Metformin as a Precision Anti-Cancer Therapy Using Structural Systems
629 Pharmacology', *Sci Rep*, 6: 20441.
- 630 Ho, Pei-Yin, Hui Li, Tengis S. Pavlov, Roland D. Tuerk, Diego Tabares, René Brunisholz, Dietbert
631 Neumann, Alexander Staruschenko, and Kenneth R. Hallows. 2018. 'β1Pix exchange factor
632 stabilizes the ubiquitin ligase Nedd4-2 and plays a critical role in ENaC regulation by AMPK in
633 kidney epithelial cells', *Journal of Biological Chemistry*, 293: 11612-24.
- 634 Hundal, Ripudaman S., Martin Krssak, Sylvie Dufour, Didier Laurent, Vincent Lebon, Visvanathan
635 Chandramouli, Silvio E. Inzucchi, William C. Schumann, Kitt F. Petersen, Bernard R. Landau,
636 and Gerald I. Shulman. 2000. 'Mechanism by which metformin reduces glucose production in
637 type 2 diabetes', *Diabetes*, 49: 2063-69.
- 638 Inyang, K. E., T. Szabo-Pardi, E. Wentworth, T. A. McDougal, G. Dussor, M. D. Burton, and T. J. Price.
639 2019. 'The antidiabetic drug metformin prevents and reverses neuropathic pain and spinal
640 cord microglial activation in male but not female mice', *Pharmacol Res*, 139: 1-16.
- 641 Laedermann, C. J., H. Abriel, and I. Decosterd. 2015. 'Post-translational modifications of voltage-
642 gated sodium channels in chronic pain syndromes', *Front Pharmacol*, 6: 263.
- 643 Laedermann, C. J., M. Cachemaille, G. Kirschmann, M. Pertin, R. D. Gosselin, I. Chang, M. Albesa, C.
644 Towne, B. L. Schneider, S. Kellenberger, H. Abriel, and I. Decosterd. 2013. 'Dysregulation of
645 voltage-gated sodium channels by ubiquitin ligase NEDD4-2 in neuropathic pain', *J Clin Invest*,
646 123: 3002-13.
- 647 Liu, Changhe, Na Hua, Xi Fu, Yilong Pan, Bin Li, and Xiaodong Li. 2018. 'Metformin Regulates the
648 Expression of SK2 and SK3 in the Atria of Rats with Type 2 Diabetes Mellitus Through the
649 NOX4/p38MAPK Signaling Pathway', *Journal of Cardiovascular Pharmacology*, 72: 205-13.
- 650 Lopez-Santiago, Luis F., Marie Pertin, Xavier Morisod, Chunling Chen, Shuangsong Hong, John Wiley,
651 Isabelle Decosterd, and Lori L. Isom. 2006. 'Sodium channel β2 subunits regulate
652 tetrodotoxin-sensitive sodium channels in small dorsal root ganglion neurons and modulate
653 the response to pain', *Journal of Neuroscience*, 26: 7984-94.
- 654 Madry, Christian, Vasiliki Kyrargyri, I. Lorena Arancibia-Cárcamo, Renaud Jolivet, Shinichi Kohsaka,
655 Robert M. Bryan, and David Attwell. 2018. 'Microglial Ramification, Surveillance, and
656 Interleukin-1β Release Are Regulated by the Two-Pore Domain K⁺ Channel THIK-1', *Neuron*,
657 97: 299-312.e6.
- 658 Mao-Ying, Q. L., A. Kavelaars, K. Krukowski, X. J. Huo, W. Zhou, T. J. Price, C. Cleeland, and C. J.
659 Heijnen. 2014. 'The anti-diabetic drug metformin protects against chemotherapy-induced
660 peripheral neuropathy in a mouse model', *PLoS One*, 9: e100701.
- 661 Melemedjian, O. K., M. N. Asiedu, D. V. Tillu, R. Sanoja, J. Yan, A. Lark, A. Khoutorsky, J. Johnson, K. A.
662 Peebles, T. Lepow, N. Sonenberg, G. Dussor, and T. J. Price. 2011. 'Targeting adenosine
663 monophosphate-activated protein kinase (AMPK) in preclinical models reveals a potential
664 mechanism for the treatment of neuropathic pain', *Mol Pain*, 7: 70.

- Melemedjian, O. K., A. Khoutorsky, R. E. Sorge, J. Yan, M. N. Asiedu, A. Valdez, S. Ghosh, G. Dussor, J. S. Mogil, N. Sonenberg, and T. J. Price. 2013. 'mTORC1 inhibition induces pain via IRS-1-dependent feedback activation of ERK', *Pain*, 154: 1080-91.
- Mia, Sobuj, Carlos Munoz, Tatsiana Pakladok, Gulab Siraskar, Jakob Voelkl, Ioana Alesutan, and Florian Lang. 2012. 'Downregulation of Kv1.5 K⁺ channels by the AMP-activated protein kinase', *Cellular Physiology and Biochemistry*, 30: 1039-50.
- Moutal, Aubin, Song Cai, Jie Yu, Harrison J. Stratton, Aude Chefdeville, Kimberly Gomez, Dongzhi Ran, Cynthia L. Madura, Lisa Boinon, Maira Soto, Yuan Zhou, Zhiming Shan, Lindsey A. Chew, Kathleen E. Rodgers, and Rajesh Khanna. 2020. 'Studies on CRMP2 SUMOylation-deficient transgenic mice identify sex-specific Nav1.7 regulation in the pathogenesis of chronic neuropathic pain', *Pain*, 161: 2629-51.
- Musi, Nicolas, Michael F. Hirshman, Jonas Nygren, Monika Svanfeldt, Peter Bavenholm, Olav Rooyackers, Gaochao Zhou, Joanne M. Williamson, Olle Ljunqvist, Suad Efendic, David E. Moller, Anders Thorell, and Laurie J. Goodyear. 2002. 'Metformin increases AMP-activated protein-kinase activity in skeletal muscle of subjects with type 2 diabetes', *Diabetes*, 51: 2074-81.
- Özdemir, Aysun, and Mustafa Ark. 2021. 'A novel ROCK inhibitor: off-target effects of metformin', *TURKISH JOURNAL OF BIOLOGY*, 45: 35-45.
- Schindelin, Johannes, Ignacio Arganda-Carreras, Erwin Frise, Verena Kaynig, Mark Longair, Tobias Pietzsch, Stephan Preibisch, Curtis Rueden, Stephan Saalfeld, Benjamin Schmid, Jean Yves Tinevez, Daniel James White, Volker Hartenstein, Kevin Eliceiri, Pavel Tomancak, and Albert Cardona. 2012. "Fiji: An open-source platform for biological-image analysis." In, 676-82. *Nat Methods*.
- Schmalhofer, William A., Jeffrey Calhoun, Rachel Burrows, Timothy Bailey, Martin G. Kohler, Adam B. Weinglass, Gregory J. Kaczorowski, Maria L. Garcia, Martin Koltzenburg, and Birgit T. Priest. 2008. 'ProTx-II, a selective inhibitor of Nav1.7 sodium channels, blocks action potential propagation in nociceptors', *Molecular Pharmacology*, 74: 1476-84.
- Shi, Peijun P., Xiao R. Cao, Eileen M. Sweezer, Thomas S. Kinney, Nathan R. Williams, Russell F. Husted, Ramesh Nair, Robert M. Weiss, Roger A. Williamson, Curt D. Sigmund, Peter M. Snyder, Olivier Staub, John B. Stokes, and Baoli Yang. 2008. 'Salt-sensitive hypertension and cardiac hypertrophy in mice deficient in the ubiquitin ligase Nedd4-2', *American Journal of Physiology - Renal Physiology*, 295.
- Sterne, Jean. 1957. 'Du nouveau dans les antidiabetiques. La NN dimethylamine guanyle guanide (NNDG)', *Maroc Medicine*, 36: 1295-96.
- Taylor, A., A. H. Westveld, M. Szkudlinska, P. Guruguri, E. Annabi, A. Patwardhan, T. J. Price, and H. N. Yassine. 2013. 'The use of metformin is associated with decreased lumbar radiculopathy pain', *J Pain Res*, 6: 755-63.
- Woods, A., K. Dickerson, R. Heath, S. P. Hong, M. Momcilovic, S. R. Johnstone, M. Carlson, and D. Carling. 2005. 'Ca²⁺/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells', *Cell Metab*, 2: 21-33.
- Woods, A., S. R. Johnstone, K. Dickerson, F. C. Leiper, L. G. Fryer, D. Neumann, U. Schlattner, T. Wallimann, M. Carlson, and D. Carling. 2003. 'LKB1 is the upstream kinase in the AMP-activated protein kinase cascade', *Curr Biol*, 13: 2004-8.
- Zhou, G., R. Myers, Y. Li, Y. Chen, X. Shen, J. Fenyk-Melody, M. Wu, J. Ventre, T. Doebber, N. Fujii, N. Musi, M. F. Hirshman, L. J. Goodyear, and D. E. Moller. 2001. 'Role of AMP-activated protein kinase in mechanism of metformin action', *Journal of Clinical Investigation*, 108: 1167-74.
- Zimmermann, Manfred. 1983. "Ethical guidelines for investigations of experimental pain in conscious animals." In, 109-10. *Pain*.

Figure Legends

Figure 1.

The effect of metformin on the $\text{Na}_v1.7$ peak current depends on the presence of NEDD4-2 in HEK293 cells. A) Representative traces of $\text{Na}_v1.7$ recordings, at the maximum peak current, from $\text{Na}_v1.7$ transfected HEK293 cells, co-transfected with different concentrations of NEDD4-2 plasmid. These data are supported by further data presenting the multiple traces, obtained at each step of the protocol, in the Extended Data Figure 1-2. Cells were incubated with culture medium for control conditions (top) or 20 mM metformin (bottom) during 12h. High [NEDD4-2] (ratio 1:1), Low [NEDD4-2] (ratio 1:0.3) and NEDD4-2CS (mutated form of NEDD4-2 with inactivated catalytic site) conditions are presented. B) Bar graphs presenting the $\text{Na}_v1.7$ peak current in the 4 conditions with control medium or 20mM metformin (n = 12-47 cells recorded/group). C) Representative blots of surface biotinylation of HEK293 cells co-transfected with $\text{Na}_v1.7$ and Low [NEDD4-2]. The upper band corresponds to the fully glycosylated form of $\text{Na}_v1.7$, while the lower band corresponds to the core glycosylated form. D) Respective quantification of $\text{Na}_v1.7$ expression in total input fraction (S0) and biotinylated fraction (S2), with control medium or metformin treatment (n = 10 /condition). Values were normalized to tubulin expression. These data are supported by further data presenting $\text{Na}_v1.7$ expression, with other concentration of NEDD4-2, in the Extended Data Figure 1-1. Data are represented as a box plot with whiskers extending from the 10th to the 90th percentiles and each dot represents one recorded cell (A) or one sample (D). Statistical analysis was performed using Unpaired t test with Welch's correction, for both patch clamp and western blot datasets (see Extended Data Table 1-2). * p < 0.05, ** p < 0.01, *** p < 0.001. Detailed references for the resources used are presented in the Extended Data Table 1-1.

Figure 2.

Metformin reduced the total I_{Na} and $\text{Na}_v1.7$ current densities in DRG neurons in a NEDD4-2-dependent manner. DRG neuronal primary cultures were incubated for 12 h with culture medium or

20 mM metformin. A) Representative traces of total I_{Na} , at the maximum peak current, recorded with a I/V protocol in DRG neurons from *Nedd4L^{fl/fl}* mice or *SNS-Nedd4L^{-/-}* mice. The traces were normalized to the capacitance of the recorded cell and expressed in pA/pF over time. These data are supported by further data presenting the multiple traces, obtained at each step of the protocol, in the Extended Data Figure 2-1. B) Quantified total I_{Na} current density in the four conditions (n = 22-28 cells recorded/condition). C) Representative traces of $Na_v1.7$ current density, at the maximum peak current, recorded with a I/V protocol. $Na_v1.7$ currents were isolated from the total I_{Na} by acute application of ProTxII at 5 nM during the recordings. The traces were normalized to the capacitance of the recorded cell and expressed in pA/pF over time. D) Quantified $Na_v1.7$ current density in the similar four conditions (n = 19-23 cells recorded/condition). Data are represented as a box plot with whiskers extending from the 10th to the 90th percentiles (min to max), each dot representing a single recorded cell. Statistical analysis was performed using Unpaired t test with Welch's correction (see Extended Data Table 1-2). * p < 0.05, ** p < 0.01, *** p < 0.001. Detailed references for the resources used are presented in the Extended Data Table 1-1.

Figure 3.

Effect of metformin on $Na_v1.7$ expression in DRG neurons. Primary cultures of DRG were incubated for 12 h with medium alone or 20 mM metformin. A) Representative blots of $Na_v1.7$ expression in total input fraction (S0) on DRG neurons from *Nedd4L^{fl/fl}* (left panel) or *SNS-Nedd4L^{-/-}* (right panel). B) Quantification of $Na_v1.7$ expression on the input fraction from DRG neurons of *Nedd4L^{fl/fl}* (n = 8 / treatment) or *SNS-Nedd4L^{-/-}* (n = 5-6 / treatment), treated or not with metformin. C) Representative blots of $Na_v1.7$ expression in biotinylated fraction (S2) on DRG neurons from *Nedd4L^{fl/fl}* (left panel) or *SNS-Nedd4L^{-/-}* (right panel). D) Quantification of $Na_v1.7$ expression on the biotinylated fraction from DRG neurons of *Nedd4L^{fl/fl}* (n = 8 / treatment) or *SNS-Nedd4L^{-/-}* (n = 6 / treatment), treated or not with metformin. Values were normalized to tubulin expression. These data are supported by further data presenting AMPK and p-AMPK expression, with or without metformin treatment, in the

767 Extended Data Figure 3-1. Data are represented as a box plot with whiskers extending from the 10th
768 to the 90th percentiles (min to max), each dot representing a single sample. Statistical analysis was
769 performed using Unpaired t test with Welch's correction (see Extended Data Table 1-2). * p < 0.05,
770 ** p < 0.01, *** p < 0.001. Detailed references for the resources used are presented in the Extended
771 Data Table 1-1.

772

773 **Figure 4.**

774 Metformin decreases NEDD4-2 phosphorylation. A) Representative blots of the total NEDD4-2
775 expression (top panel) and the phosphorylated NEDD4-2 (at Serine 328) (bottom panel) in DRG
776 neurons incubated with culture medium or with metformin. B) Quantification of the metformin
777 effect on NEDD4-2 expression (n = 4 / treatment) and phosphorylated NEDD4-2 (Ser328 residue) (n =
778 4 / treatment). For all datasets, values were normalized to tubulin expression and the data are
779 represented as a box plot with whiskers extending from the 10th to the 90th percentiles (min to max),
780 each dot representing a single sample. Statistical analysis was performed using Unpaired t test with
781 Welch's correction (see Extended Data Table 1-2). * p < 0.05, ** p < 0.01, *** p < 0.001. Detailed
782 references for the resources used are presented in the Extended Data Table 1-1.

783

784 **Figure 5.**

785 Current clamp recordings of DRG neurons from *Nedd4L^{fl/fl}* or *SNS-Nedd4L^{-/-}*, treated with medium or
786 20 mM metformin. The recordings are acquired from at least three different patch clamp sessions. A)
787 Representative traces of APs in different conditions elicited by ramp protocols of 500 ms (inset). B)
788 Box plots showing the analysis of APs number in *Nedd4L^{fl/fl}* (top panel, n = 14-16 / treatment)) and
789 *SNS-Nedd4L^{-/-}* mice (bottom panel, n = 11-16 / treatment); data are represented as a box plot with
790 whiskers extending from the 10th to the 90th percentiles (min to max), each dot representing a single
791 recorded cell. Statistical analysis was performed using a two-way Repeated Measures ANOVA,
792 followed by Sidak's multiple comparisons post hoc test (* p < 0.05, ** p < 0.01, *** p < 0.001) (see
793 Extended Data Table 1-2). C) Representative single AP and its parameters in different conditions as

794 illustrated by the color code. D) Analysis of AP parameters which are significantly changed upon
 795 metformin treatment in *Nedd4L^{fl/fl}* (n = 14 cells / treatment) or *SNS-Nedd4L^{-/-}* (n = 10-16 cells /
 796 treatment); data are represented as a box plot with whiskers extending from the 10th to the 90th
 797 percentiles (min to max). These data are supported by further data presenting the AP characteristics,
 798 obtained from DRG neurons of *Nedd4L^{fl/fl}* or *SNS-Nedd4L^{-/-}* treated with or without metformin, in the
 799 Extended Data Figure 5-1. Statistical analysis was performed using Unpaired t test with Welch's
 800 correction (see Extended Data Table 1-2). * p < 0.05, ** p < 0.01, *** p < 0.001. Detailed references
 801 for the resources used are presented in the Extended Data Table 1-1.

802

803 **Table 1.**

804 Biophysical properties of Na_v1.7-mediated currents in HEK293 cells, transfected with different
 805 NEDD4-two plasmids, with (+) or without (-) metformin treatment. The three groups correspond to
 806 HEK293 cells transfected 1) only with Na_v1.7, 2) with Na_v1.7 and NEDD4-2 in low concentration
 807 (Na_v1.7 cDNA/NEDD4-2 cDNA ratio 1:0.3) or 3) with Na_v1.7 and an inactivated NEDD4-2, mutated on
 808 the catalytic site. The V_½ (mV) is the voltage at which half of the available channels are activated or
 809 inactivated based on their steady-state protocol. Data are expressed as Mean ± SEM. Statistical
 810 analysis was performed using Unpaired t test with Welch's correction (see Extended Data Table 1-2).
 811 * p < 0.05, ** p < 0.01, *** p < 0.001. Detailed references for the resources used are presented in the
 812 Extended Data Table 1-1.

813

814 **Table 2.**

815 Biophysical properties of total Na_v and Na_v1.7-mediated currents in DRG neurons from *Nedd4L^{fl/fl}* or
 816 *SNS-Nedd4L^{-/-}* mice, with (+) or without (-) metformin treatment. The steady-state activation was
 817 measured on total I_{Na} current density or Na_v1.7-mediated currents for each genotype and treatment
 818 condition. The V_½ (mV) is the voltage at which half of the available channels are activated with the
 819 steady-state activation protocol. Data are expressed as Mean ± SEM. Statistical analysis was
 820 performed using Unpaired t test with Welch's correction (see Extended Data Table 1-2). * p < 0.05,

821 ** $p < 0.01$, *** $p < 0.001$. Detailed references for the resources used are presented in the Extended
 822 Data Table 1-1.

823

824 [Extended Data](#)

825

826 **Extended Data Figure 1-1.**

827 Supplemental data Figure supporting the Figure 1. Effect of metformin on Na_v1.7 expression in
 828 HEK293 cells, depending on NEDD4-2 expression levels. A) Representative blots of the total input
 829 fraction (S0) or surface biotinylation (S2) of HEK293 cells co-transfected with Na_v1.7 and no [NEDD4-
 830 2], treated with medium or metformin. B) Respective quantification of Na_v1.7 expression in total
 831 input fraction (S0, left panel) and biotinylated fraction (S2, right panel), with control medium or
 832 metformin treatment (n = 6 / condition). C) Representative blots of the total input fraction (S0) or
 833 surface biotinylation (S2) of HEK293 cells co-transfected with Na_v1.7 and high [NEDD4-2], treated
 834 with control medium or metformin. D) Respective quantification of Na_v1.7 expression in total input
 835 fraction (S0, left panel) and biotinylated fraction (S2, right panel), with control medium or metformin
 836 treatment (n = 6 / condition). Values were normalized to tubulin expression. Data are represented as
 837 a box plot with whiskers extending from the 10th to the 90th percentiles; each dot represents one
 838 sample. Statistical analysis was performed using unpaired t-test with Welch's correction (see
 839 Extended Data Table 1-2). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Detailed references for the resources
 840 used are presented in the Extended Data Table 1-1.

841

842 **Extended Data Figure 1-2.**

843 Supplemental data Figure supporting the Figure 1. Representative example traces of Na_v1.7 currents,
 844 at each step of the protocol. The voltage clamp protocol used is represented below the traces. It
 845 consisted in evoked sodium currents, at a holding potential of -100 mV, with pulses of 100 ms
 846 ranging from -80 to +40 mV in increments of 5 mV. Recordings from transfected HEK293 cells, co-
 847 transfected with Na_v1.7 and different concentrations of NEDD4-2 plasmid. Cells were incubated with

848 culture medium for control conditions (top) or 20 mM metformin (bottom) during 12h. High [NEDD4-
 849 2] (ratio 1:1), Low [NEDD4-2] (ratio 1:0.3) and NEDD4-2CS (mutated form of NEDD4-2 with
 850 inactivated catalytic site) conditions are presented. Detailed references for the resources used are
 851 presented in the Extended Data Table 1-1.

852

853 **Extended Data Figure 2-1.**

854 Supplemental data Figure supporting the Figure 2. Representative example traces of total I_{Na} or
 855 $Na_v1.7$ currents, at each step of the protocol, recorded from different DRG neurons, with or without
 856 metformin treatment. The voltage clamp protocol used is represented below the traces. It consisted
 857 in evoked sodium currents, at a holding potential of -100 mV, with pulses of 100 ms ranging from -80
 858 to +40 mV in increments of 5 mV. A) Recordings of total I_{Na} from DRG neurons of *Nedd4-L^{f/f}* mice or
 859 *SNS-Nedd4-L^{-/-}* mice. The traces were normalized to the capacitance of the recorded cell and
 860 expressed in pA/pF over time. B) Recordings of $Na_v1.7$ current density from DRG neurons of *Nedd4-*
 861 *L^{f/f}* mice or *SNS-Nedd4-L^{-/-}* mice. $Na_v1.7$ currents were isolated from the total I_{Na} by acute application
 862 of ProTxII at 5 nM during the recordings. The traces were normalized to the capacitance of the
 863 recorded cell and expressed in pA/pF over time. Detailed references for the resources used are
 864 presented in the Extended Data Table 1-1.

865

866 **Extended Data Figure 3-1.**

867 Supplemental data Figure supporting the Figure 3. Effect of metformin on AMPK and AMPK
 868 phosphorylation (p-AMPK) in HEK293 cells and DRG neurons. A) Representative blots of AMPK and p-
 869 AMPK expression in HEK293 cells, treated with medium or 20 mM metformin. B) Respective
 870 quantification of AMPK, p-AMPK expressions, and p-AMPK/AMPK ratio (n = 6 / condition). C)
 871 Representative blots of AMPK and p-AMPK expression in DRG neurons, treated with control medium
 872 or 20 mM metformin. D) Respective quantification of AMPK, p-AMPK expressions, and p-
 873 AMPK/AMPK ratio (n = 6 / condition). Values were normalized to tubulin expression. Data are
 874 represented as a box plot with whiskers extending from the 10th to the 90th percentiles; each dot

875 represents one sample. Statistical analysis was performed using unpaired t-test with Welch's
 876 correction (see Extended Data Table 1-2). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Detailed references
 877 for the resources used are presented in the Extended Data Table 1-1.

878

879 **Extended Data Figure 5-1.**

880 Supplemental data Table supporting the Figure 5. AP characteristics recorded from DRG neurons by
 881 electrophysiological current clamp protocol. Neurons were obtained from *Nedd4L^{fl/fl}* (n = 14 cells /
 882 treatment) or *SNS-Nedd4L^{-/-}* (n = 10–16 cells / treatment) mice and treated with control medium or
 883 metformin treatment (20 mM). Data are represented as means $\pm$ SEM. Statistical analysis was
 884 performed using Unpaired t-test with Welch's correction (see Extended Data Table 1-2). * $p < 0.05$,
 885 ** $p < 0.01$, *** $p < 0.001$. Detailed references for the resources used are presented in the Extended
 886 Data Table 1-1.

887

888 **Extended Data Table 1-1.**

889 Supplemental data Table of reagents, solutions and RRID references, supporting all the Figures. RRID
 890 and source references of the resources used in the current study.

891

892 **Extended Data Table 1-2.**

893 Supplemental data Table of all statistical analysis, supporting all the Figures. Statistical table. Each
 894 row represents a dataset and the corresponding statistical tests, statistical values, p values, and
 895 Cohen's effect size factor.

Table 1

	Na _v 1.7 alone		Na _v 1.7 + Low [NEDD4-2]		Na _v 1.7 + N
Metformin	-	+	-	+	-
Steady-state activation					
V _{1/2} (mV)	-8.1 ± 0.7	-6.3 ± 1.1	-6.7 ± 1.1	-5.4 ± 1.4	-9.3 ± 0.6
Slope	6.5 ± 0.2	7.1 ± 0.3	6.0 ± 0.3	** 7.2 ± 0.3	7.1 ± 0.3
n	30	17	16	11	12
Steady-state inactivation					
V _{1/2} (mV)	-61.3 ± 1.1	-61.5 ± 1.3	-58.0 ± 1.5	-58.9 ± 1.8	-65.8 ± 1.2
Slope	7.45 ± 0.4	* 9.01 ± 0.6	8.82 ± 0.7	7.75 ± 0.6	7.72 ± 0.5
n	30	16	15	14	12

EDD4-2CS
+
-9.1 ± 0.9
7.1 ± 0.2
11
-65.2 ± 1.3
7.1 ± 0.3
12

Table 2

	Nedd4-2 ^{fl/fl}				SNS-Nedd4-2 ^{-/-}		
	Na _v total		Na _v 1.7		Na _v total		Na
Metformin	-	+	-	+	-	+	-
Steady-state activation							
V _{1/2} (mV)	-37.1 ± 2.3	-35.6 ± 3.4	-42.8 ± 3.2	-40.4 ± 4.7	-37.0 ± 2.7	-39.9 ± 4.8	-36.5 ± 14.0
Slope	6.5 ± 0.5	6.6 ± 0.6	5.9 ± 1.0	5.2 ± 1.2	6.2 ± 0.4	**8.2 ± 0.4	7.0 ± 1.5
n	15	10	6	5	12	8	3

$\sqrt{1.7}$
+
-43.2 ± 4.9
5.9 ± 0.6
6