# A novel gain-of-function sodium channel β2 subunit mutation in idiopathic small fiber neuropathy

Matthew Alsaloum[1,2,3,4,5], Julie Labau[1,2,3,6,9], Daniel Sosniak[1,2,3], Peng Zhao[1,2,3], Rowida Almomani[6,7], Monique Gerrits[8], Janneke G.J. Hoeijmakers[9], Giuseppe Lauria[10,11], Catharina G. Faber[9], Stephen G. Waxman[1,2,3], Sulayman Dib-Hajj[1,2,3]

[1]Department of Neurology, Yale University School of Medicine, New Haven, CT, USA.

[2]Center for Neuroscience & Regeneration Research, Yale University, West Haven, CT, USA.

[3]Center for Rehabilitation Research, VA Connecticut Healthcare System, West Haven, CT, USA.

[4]Yale Medical Scientist Training Program, Yale School of Medicine, New Haven, CT, USA.

[5]Interdepartmental Neuroscience Program, Yale School of Medicine, New Haven, CT, USA.

[6]Department of Genetics and Cell Biology, Clinical Genomics Unit, Maastricht University, Maastricht, the Netherlands

[7]Department of Medical Laboratory Sciences, Jordan University of Science and Technology, Irbid, Jordan

[8]Department of Clinical Genetics, Maastricht University Medical Centre+, Maastricht, the Netherlands

[9]Department of Neurology, Maastricht University Medical Centre+, Maastricht, the Netherlands

[10]Neuroalgology Unit, IRCCS Foundation "Carlo Besta" Neurological Institute, Milan, Italy

[11]Department of Biomedical and Clinical Sciences "Luigi Sacco," University of Milan, Milan, Italy


Corresponding author:
Sulayman D. Dib-Hajj, PhD
Neuroscience and Regeneration Research Center
VA Connecticut Healthcare System
950 Campbell Avenue, Bldg. 34
West Haven, CT 06516
Email: sulayman.dib-hajj@yale.edu

1

## Abstract

Small fiber neuropathy (SFN) is a common condition affecting thinly myelinated Aδ and unmyelinated C fibers, often resulting in excruciating pain and dysautonomia. SFN has been associated with several conditions, but a significant number of cases have no discernible cause. Recent genetic studies have identified potentially pathogenic gain-of-function mutations in several the pore-forming voltage-gated sodium channel α subunits ($Na_V$s) in a subset of patients with SFN, but the auxiliary sodium channel β subunits have been less implicated in the development of the disease. β subunits modulate $Na_V$ trafficking and gating, and several mutations have been linked to epilepsy and cardiac dysfunction. Recently, we provided the first evidence for the contribution of a mutation in the β2-subunit to pain in human painful diabetic neuropathy.

Here, we provide the first evidence for the involvement of a sodium channel β subunit mutation in the pathogenesis of SFN with no other known causes. We show, through current-clamp analysis, that the newly-identified Y69H variant of the β2 subunit induces neuronal hyperexcitability in dorsal root ganglion neurons, lowering the threshold for action potential firing and allowing for increased repetitive action potential spiking. Underlying the hyperexcitability induced by the β2-Y69H variant, we demonstrate an upregulation in tetrodotoxin-sensitive, but not tetrodotoxin-resistant sodium currents. This provides the first evidence for the involvement of β2 subunits in SFN and strengthens the link between sodium channel β subunits and the development of neuropathic pain in humans.

2

**New & Noteworthy**

Small fiber neuropathy (SFN) often has no discernible cause, although mutations in the voltage-gated sodium channel α-subunits have been implicated in some cases. We identify a patient suffering from SFN with a mutation in the auxiliary β2-subunit and no other discernible causes for SFN. Functional assessment confirms this mutation renders dorsal root ganglion neurons hyperexcitable and upregulates tetrodotoxin-sensitive sodium currents. This study strengthens a newly-emerging link between sodium channel β2-subunit mutations and human pain disorders.

3

**Introduction**

Small-fiber neuropathy (SFN) is a painful condition selectively affecting thinly
myelinated Aδ-fibers and unmyelinated C-fibers[1]. Clinically, SFN often presents with
chronic pain, usually described as "burning" and in a "glove and stocking" distribution,
dysautonomic symptoms, no signs of large-diameter fiber involvement, and reduced
intraepidermal nerve fiber density on skin biopsy[2-5]. The sequelae of SFN are
associated with significant reductions in patient quality of life[6]. Additionally, worsening
SFN pain is also directly associated with worse mental health, sleep, and employment
outcomes[7]. Current first-line therapeutic strategies for the management of chronic
pain associated with painful neuropathy, including SFN, are not very effective, in part
due to adverse effects[8, 9]. More effective pharmacological management is thus a high
priority, and a better understanding of underlying causes of pain may provide more
effective treatment options.

Many causes of SFN have been identified[10-12], including autoimmune conditions,
sodium channel gene mutations[1, 13-16], diabetes mellitus[17], and
chemotherapy[18]. However, a significant proportion of patients with SFN have no
identifiable cause, termed idiopathic small-fiber neuropathy (I-SFN)[10, 19]. Knowledge
of the etiology underlying SFN is important as some conditions are preventable or can
be targeted with a precision medicine approach[20].

Mutations in sodium channel genes are responsible for a sizable percentage of SFN
cases (11.6 %)[16], which is explicable as voltage-gated sodium channels ($Na_V$s) play a
pivotal role in regulating neuronal excitability[21, 22]. Sodium channels are composed
of a pore-forming α-subunit ($Na_V$) which is associated with auxiliary β subunits[23]. Nine

4

93   different Na$_V$s (Na$_V$1.1-Na$_V$1.9) are expressed in humans(24). Na$_V$1.7, Na$_V$1.8, and

94   Na$_V$1.9 are preferentially expressed in the developed peripheral nervous system and

95   have been genetically and functionally well-validated as drivers of chronic pain in

96   humans(25-27). Na$_V$1.7 was the first Na$_V$ channel linked directly to pain, with gain-of-

97   function mutations resulting in a variety of pain conditions, including inherited

98   erythromelalgia(28-32), paroxysmal extreme pain disorder, and SFN(1). Loss-of-

99   function mutations result in congenital insensitivity to pain(33-35). Subsequently, other

100  Na$_V$s were found to play pivotal roles in SFN, including both Na$_V$1.8(13, 36) and

101  Na$_V$1.9(15). However, while the contribution of Na$_V$s to painful neuropathies has been

102  thoroughly investigated, the contributions of sodium channel β subunits to pain are still

103  being elucidated.

104  The sodium channel β-subunit family is comprised of four genes, *SCN1B-SCN4B*,

105  encoding four distinct proteins, β1-β4, and two splice variants, β1A and β1B(37, 38).

106  Na$_V$s are found as heterotrimeric complexes *in vivo*, consisting of one pore-forming α-

107  subunit (Na$_V$1.1-Na$_V$1.9) and two non-ion conducting β-subunits (β1/3 and β2/4)(39, 40).

108  β-subunits play important roles in a number of cellular and molecular processes. For

109  example, β-subunits are known to alter the biophysical properties of multiple Na$_V$ α-

110  subunits(41-44). They also are important for normal Na$_V$ localization and membrane

111  trafficking(44-49). Additionally, β-subunits act as cell adhesion molecules(50) and are

112  implicated in multiple diseases of the cardiac conducting system(51, 52) and

113  epilepsy(53-56). Given the multiple roles of sodium channel β-subunits in Na$_V$

114  modulation, it is unsurprising that their upregulation has been linked to neuropathic pain,

115  while their knock-out or knockdown ameliorates pain in preclinical rodent models(57,

5

116    58). Only recently, though, has a mutation in a β-subunit (β2-D109N) been linked to

117    pain in a patient with painful diabetic neuropathy(59). Functional analysis showed that

118    the D109N mutation caused a depolarizing shift in the voltage-dependence of $Na_v1.7$

119    fast-inactivation and reduced use-dependent inhibition of the $Na_v1.7$ channel, enhancing

120    dorsal root ganglion (DRG) neuronal action potential firing, consistent with its

121    contribution to pain in this patient. Here, we present the case of a novel β2-subunit

122    mutation involved in the development small-fiber neuropathy with no other underlying

123    pathophysiology. The newly identified Y69H variant increases tetrodotoxin-sensitive

124    (TTX-S) current density, without altering $Na_v$ gating properties, enhancing the

125    excitability of DRG neurons. Our data expands the role of β2 subunit mutations at the

126    sodium channel level and strengthens the evidence for a role of these subunits in

127    human pain disorders.

128

6

**Materials and Methods**

*DNA isolation from peripheral blood*

Local medical ethical committees at each of the participating centers approved this study. Written informed consent by patients was obtained prior to participation in this study. A peripheral blood sample was taken from all patients in a cohort with SFN[59] and genomic DNA was extracted from peripheral blood using either a QIAamp DNA Blood Maxi Kit/Puregene Blood Core Kit (Qiagen, Hilden, Germany) or a NucleoSpin 8 Blood Isolation Kit (Macherey-Nagel, Düren, Germany). Quality and concentration of the DNA were determined by NanoDrop (Thermo Scientific, Wilmington, DE, USA) and Qubit 2.0 Fluorometer using the Qubit® dsDNA BR assay kit (Life technologies, Bleiswijk, The Netherlands). Isolated DNA was stored with a unique numeric code in the central DNA bank at Maastricht University Medical Centre and IRCCS Foundation "Carlo Besta" Neurological Institute.

*Single-molecule molecular inversion probe-next-generation sequencing*

Genetic sequencing was conducted as previously described[60]. In brief, coding exons and exon-flanking intron sequences (±20bp) of *SCN1B-4B*, *SCN3A*, and *SCN7A-11A* were sequenced by single-molecule molecular inversion probe-next-generation sequencing (smMIP-NGS). Three-hundred-twenty-four smMIPs were designed using a modified version of MIPgen software (http://shendurelab.github.io/MIPGEN/). The gap fill length between the extension and ligation arm (region of interest) of the smMIPs was fixed to 220–230 nucleotide. Probes were synthesized by Integrated DNA Technologies (IDT, Iowa, IA, USA).

7

151     To identify sequence variations in *SCN1B-4B*, *SCN3A*, *SCN7A-11A*, patients' coding

152     and immediate flanking regions of these genes were compared with reference

153     sequence GRCh37. Genetic variations detected were annotated according to the

154     guidelines of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/).

155     Variants with a possible pathogenic effect were classified using Alamut Mutation-

156     Interpretation Software (Interactive-Biosoftware, Rouen, France). Classification of

157     variants was based on the practice guidelines of the Association for Clinical Genetic

158     Science.

159     *Isolation and transfection of DRG neurons*

160     The Y69H mutation was introduced into the plasmid encoding wild-type human-β2-

161     IRES-GFP(61) using QuickChange Lightening site-directed mutagenesis (Agilent, Santa

162     Clara, CA).  All animal studies and procedures followed a protocol that was approved by

163     the Veterans Administration Connecticut Healthcare System Institutional Animal Care

164     and Use Committee. DRG neurons from 4- to 6- week old Sprague-Dawley rats of both

165     sexes were harvested and dissociated as described previously with minor

166     differences(62). In brief, adult rat DRGs were dissociated with a 20- minute incubation in

167     1.5 mg/mL collagenase A (Roche, Indianapolis, IN, USA) and 0.6 mM EDTA, followed

168     by a 17-minute incubation in 1.5 mg/mL collagenase D (Roche), 0.6 mM EDTA, and 30

169     U/mL papain (Worthington Biochemical, Lakewood, NJ, USA). DRGs were then

170     centrifuged and triturated in 0.5 mL of DRG media (DMEM/F12 with 100 U/ml penicillin,

171     0.1 mg/ml streptomycin, and 10% fetal bovine serum) containing 1.5 mg/mL bovine

172     serum albumin (low endotoxin) and 1.5 mg/mL trypsin inhibitor (Sigma, St. Louis, MO,

173     USA). After trituration, undigested tissue was filtered using a 70-μm cell strainer (Becton

8

174　Dickinson, Franklin Lakes, NJ, USA). The mesh was washed twice with 2 mL of DRG

175　media. Neurons were then pelleted and transfected with either human-β2-Y69H or

176　human-β2-wild type (WT) cDNA carrying an internal ribosome entry site (IRES)-GFP to

177　mark transfected cells using a Nucleofector IIS (Lonza, Basel, Switzerland) and Amaxa

178　Basic Neuron SCN Nucleofector Kit (VSPI-1003).

179　*HEK293 stable cell line transfection*

180　Human embryonic kidney (HEK293) cells stably expressing a tetrodotoxin-resistant

181　version of the $Na_V1.7$ channel were transfected with either 0.5 μg/μL of human β2-Y69H

182　or human β2-WT cDNA containing an internal ribosome entry site (IRES)-GFP tag using

183　a LipoJet transfection kit (SignaGen Laboratories, Rockville, MD, USA). The cells were

184　grown and maintained under standard culture conditions ($37^oC$, 5% CO2) in Dulbecco's

185　modified Eagle's medium (DMEM/F12), supplemented with 10% fetal bovine serum

186　(FBS) and 1% penicillin/streptomycin. Transfected cells were grown in 35 mm dishes,

187　before being resuspended and plated onto PDL/laminin-coated coverslips the next day.

188　Sodium currents were recorded 24-48 hours following transfection.

189　*Macroscopic current recordings*

190　Macroscopic currents were recorded from adult rat DRG neurons in both current-clamp

191　and voltage-clamp configurations using an EPC-10 amplifier and the PatchMaster

192　program (HEKA Elektronik, Holliston, MA, USA) at room temperature. Patch pipettes

193　were pulled from borosilicate glass (1.65 mm/1.1 mm outside diameter/inside diameter;

194　World Precision Instruments, Sarasota, FL, USA) using a Sutter Instruments P-97 puller

195　and had a resistance of 0.6-1.8 MΩ.

196    For current-clamp recordings, the extracellular solution contained (in mM): 140 NaCl, 3

197    KCl, 2 $MgCl_2$, 2 $CaCl_2$, and 10 HEPES. Patch pipettes were filled with intracellular

198    solution containing (in mM): 140 KCl, 3 Mg-ATP, 0.5 EGTA, 5 HEPES, and 30 dextrose.

199    Both solutions were titrated to a pH of 7.3 with NaOH and KOH, respectively, and

200    brought to a final osmolarity (320 and 310 mOsm for extracellular and intracellular

201    solutions, respectively) using dextrose. Whole-cell configuration was obtained in

202    voltage-clamp mode before transitioning to current-clamp mode. Fluorescent small DRG

203    neurons (<30 µm diameter) with stable (<10% variation) resting membrane potentials

204    (RMPs) and minimal leak currents at break-in were included in the analysis. Cells with

205    an input resistance lower than 100 MΩ were excluded from analysis. Input resistance

206    was calculated as the slope of the linear fit of the hyperpolarizing response to current

207    steps from -5 pA to -40 pA in 5 pA increments.

208    In current-clamp mode, rheobase was defined as the first current injection step that

209    resulted in action potential firing without further failure and was determined by a series

210    of depolarizing current injections (200 ms) that increased incrementally by 5 pA. For the

211    calculation of rheobase, action potentials were defined as rapid increases in membrane

212    potential to >40 mV with a total amplitude >80 mV. However, as neurons often

213    attenuate firing with repetitive action potential spiking, when examining repetitive firing,

214    action potentials were counted if the membrane potential rapidly crossed 0 mV,

215    regardless of overshoot or total amplitude. Action potential repetitive firing frequency

216    was determined by quantifying the number of action potentials that a neuron fired during

217    a 500 ms current injection at current injections between 25 and 500 pA, in 25 pA steps.

10

218  For voltage-clamp recordings, the extracellular bath solution contained (in mM) 140

219  NaCl, 20 TEA-Cl, 3 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 5 CsCl, 0.1 CdCl$_2$, and 10 HEPES (± 0.1

220  tetrodotoxin [TTX]), titrated to a pH of 7.3 using NaOH. Patch microelectrodes contained

221  an intracellular solution consisting of (in mM): 140 CsF, 10 NaCl, 1.1 EGTA, and 10

222  HEPES, titrated to a pH of 7.3 using CsOH. Both solutions were brought to final

223  osmolality (320 mOsm for extracellular solution and 310 mOsm for intracellular solution)

224  using dextrose.

225  Sodium currents from fluorescent (indicating successful transfection) DRG neurons

226  under 30 μm in diameter were recorded in the whole-cell configuration. For biophysical

227  analysis of sodium channel gating, series resistance prediction and compensation

228  (60%-90%) were applied to reduce the voltage errors. Neurons with a peak voltage

229  error greater than 5 mV were excluded from all biophysical analyses. The recorded

230  currents were digitized at a rate of 50 kHz after passing through a low-pass Bessel filter

231  setting of 10 kHz. After achieving whole-cell configuration, a 5-minute equilibration

232  period was allowed before starting the recording. DRGs were held at -100 mV to

233  minimize inactivation of Na$_V$1.9 while maintaining neuronal cell viability.

234  To measure sodium channel activation, DRG neurons were pulsed to a range of

235  potentials between -80 mV and +30 mV, in 5 mV increments, for 100 ms after being

236  held at a holding potential of -100 mV. Peak inward currents were transformed to

237  conductance using the equation $G = \frac{I_{Na}}{V_m - E_{Na}}$. Reversal potentials were calculated in the

238  FitMaster program (HEKA Elektronik). The conductance at each voltage was

239  normalized to the maximum conductance and fit with the following Boltzmann equation

11

240  to derive the activation curve: $G = \frac{G_{min}+(G_{max}-G_{min})}{1+e^{((V_{0.5}-V_m)/K)}}$, where $V_{0.5}$ is the half-maximal

241  activation potential and k is the slope factor of the activation curve.

242  Use-dependent inhibition at 20 Hz stimulation was determined by holding the neurons at

243  -80 mV for 2 ms, followed by a 10 ms step to −20 mv and measuring the charge transfer

244  through the cell. Use-dependence curves were generated by normalizing the current

245  passed at each sweep to the current passed at the first sweep.

246  To assess steady-state fast-inactivation, DRG neurons were prepulsed to a range of

247  voltages between −140 mV and 0 mV in 10 mV steps for 500 ms from a holding potential

248  of −100 mV and then pulsed to a potential of −10 mV. Peak inward current was

249  normalized to the maximum peak inward current and fit with the following double

250  Boltzmann equation to derive the inactivation curve:

251  $I = I_{min} + A[\frac{p}{1+e^{\frac{x-x_{01}}{k_1}}} + \frac{1-p}{1+e^{\frac{x-x_{02}}{k_2}}}]$.

252  To assess deactivation, DRG neurons were activated by a brief 0.5 ms pulse to -10 mV

253  from a holding potential of -120 mV. Following this brief activation step, the kinetics of

254  deactivation were examined by 50 ms pulses at a range of potentials from -40 mV to -

255  120 mV in -5 mV increments. The tail-currents were fit with a single exponential

256  equation in order to derive the time constant of decay.

257  To assess recovery from fast-inactivation, we utilized a two-pulse protocol (both to -10

258  mV) with varied interpulse interval between 1 and 513 ms at voltage potentials between

259  -120 mV and -70 mV. The current evoked by the test pulse at -10 mV was normalized to

260  the current elicited by the prepulse at -10 mV to represent the fraction of recovering

12

261 currents. This was then plotted against the interpulse duration to calculate the rate and

262 extent of recovery from inactivation.

263 Peak current density was calculated as the peak current during the activation pulse

264 (described above) normalized to the cellular capacitance. To assess tetrodotoxin-

265 resistant (TTX-R) and TTX-S currents, 1 µM tetrodotoxin (TTX) was applied. Total

266 sodium currents were recorded prior to TTX application. Extracellular bath solution

267 containing 1 µM TTX was subsequently perfused into the chamber while continuously

268 suctioning the chamber. In order to ensure full solution exchange, at least 5X the total

269 volume of the chamber of solution was perfused. Additionally, test pulses to -10 mV

270 were conducted until the current no longer decreased. TTX-R currents were

271 subsequently recorded. To calculate TTX-S currents, reference series subtraction in

272 PatchMaster was utilized.

273 *Molecular modeling*

274 Protein data bank (PDB) structure 6J8I from Shen *et al.(39)* was downloaded from

275 RCSB.org and embedded in a heterogeneous lipid bilayer using CHARMM-GUI(63) to

276 reflect a more physiologically relevant background. The structure was then edited in

277 PyMol (Schrödinger, LLC).

278 *Data Analysis*

279 All data are represented as mean ± standard error of the mean, unless otherwise noted.

280 All Boltzmann fits and graphical representation was conducted in GraphPad Prism.

281 Significance in figures is noted as * ($p \leq 0.05$), ** ($p \leq 0.01$), or *** ($p \leq 0.001$).

282

13

**Results**

*Identification of a novel variant in the coding region of the SCN2B gene*

In our cohort of 548 patients diagnosed with painful SFN and no other underlying

causes, MIPs-NGS identified two potentially pathogenic novel *SCN2B* gene variants

that were found only in patients with painful SFN and not in any patients with painless

conditions. Of these variants, one (c.502G>T; pG168C) was in the transmembrane

segment of β2, whereas the second (c.205T>C; pY69H), a tyrosine-to-histidine

missense mutation at position 69 resides in the immunoglobulin domain of the protein

(Figure 1). The rare Y69H variant, classified as a variant of unknown significance(64)

with an allele frequency of $4.95 \times 10^{-5}$, was initially chosen for investigation because it

had also been identified in patients with Brugada syndrome and one patient with chronic

atrial fibrillation(65). Additionally, the only previously characterized mutation in the

sodium channel β2 subunit associated with a painful neuropathy was also located in the

immunoglobulin domain(59).

The β2-Y69H variant was identified in a patient with painful idiopathic SFN presenting

as a stabbing and burning pain in both legs, as well as red discoloration of the soles of

her feet, beginning at age 53. She was assessed clinically and her neurologic physical

exam was normal. She also had normal nerve conduction studies, consistent with

unaffected large, myelinated nerve fibers. Thermal testing showed abnormal warmth

and cold sensation, and reduced intraepidermal nerve fiber density (1.1 per mm, with a

lower limit of normal accepted at 4.3 per mm). There was no other relevant medical

history that could potentially explain her peripheral neuropathy. Cardiovascular workup

was normal and no prior history of cardiovascular disease was noted.

14

306    Electrocardiogram conducted at the time showed sinus rhythm with normal conduction

307    times; the PR interval spanned 154 ms (normal limits: 120-200 ms), the QRS complex

308    spanned 86 ms (normal limits: 60-100 ms), and the QT interval spanned 464 ms, with a

309    corrected QT (QTc) interval of 401 ms (99th percentile QTc in post-pubertal females:

310    480 ms). No other *SCN1B-4B*, *SCN3A*, or *SCN7A-11A* variants, others than the *SCN2B*

311    Y69H variant, were detected by MIPs-NGS; she was heterozygous for the *SCN2B*

312    Y69H variant.

313    *Human β2-Y69H confers hyperexcitability on DRG neurons*

314    To test whether the Y69H variant contributes to enhanced neuronal excitability, DRG

315    neurons were isolated from adult rats and transfected with either wild-type human β2 or

316    the β2-Y69H variant. Excitability of DRG neurons was analyzed by current-clamp

317    recordings. We examined passive membrane properties, such as the neuronal RMP

318    (Figure 2A). The RMP of neurons expressing the β2-Y69H variant (-48.21 ± 1.99 mV, n

319    = 11) was not statistically different from that of neurons expressing the wild-type β2

320    subunit (-50.18 ± 2.48 mV, n = 11, two-tailed Student's t-test p = 0.54). Additionally,

321    input resistances of non-spontaneously firing DRG neurons were comparable between

322    those expressing the wild-type β2 subunit (732.95 ± 130.13 MΩ, n = 11) and those

323    expressing the Y69H variant (959.70 ± 97.82 MΩ, n = 11, Student's t-test p = 0.18).

324    Neurons expressing the β2-Y69H variant were more excitable at rest than neurons

325    exhibiting the wild-type β2 subunit (Figure 2B), with a numerically larger percentage of

326    neurons firing spontaneous action potentials; 47.62% of neurons expressing the Y69H

327    variant fired action potentials spontaneously, compared to only 21.42% of neurons

328    expressing the wild-type β2 subunit. However, this study was not powered to detect

15

329     spontaneous action potential firing and was therefore not statistically significant

330     (Fisher's exact test, p = 0.16).

331     Neurons expressing the β2-Y69H variant are significantly more excitable in response to

332     current injection stimuli than neurons expressing the wild-type β2 subunit. We examined

333     the current injection necessary to evoke an action potential in adult rat DRG neurons

334     (Figure 3A, 3B). Rheobase (Figure 3B) was significantly lower in neurons expressing

335     the Y69H variant (51.36 ± 53.81 pA, n = 11) compared to neurons expressing the wild-

336     type β2 subunit (163.64 ± 47.85 pA, n = 11, Student's t-test, p = 0.038). Furthermore,

337     neurons expressing the Y69H variant were capable of significantly increased repetitive

338     action potential firing, compared to neurons expressing the wild-type β2 variant (Figure

339     3C, 3D, 3E). When each neuron was injected with a 1-second current stimulus at the

340     amplitude that was sufficient to stimulate an action potential at 200-millisecond duration,

341     to the nearest 25 pA, neurons expressing the wild-type β2 subunit fired significantly

342     fewer action potentials (0.73 ± 0.47, n = 11) than did neurons expressing the Y69H

343     variant (3.82 ± 1.01, n = 11, two-tailed Student's t-test p = 0.0065, Figure 3C). In

344     response to graded current injections from 25 pA to 500 pA in 25 pA increments,

345     neurons expressing the Y69H variant fired significantly more action potentials across

346     the entire stimulus range (two-way repeated measures ANOVA, p = 0.004). This

347     difference was statistically significant after correcting for multiple comparisons using the

348     Holm-Šídák method at 150, 175, 200, 225, and 275 pA current injections (Figure 3E).

349     *Biophysical analysis of the β2-Y69H variant on total sodium current*

350     We investigated mechanisms underlying the hyperexcitability of DRG neurons

351     expressing the β2-Y69H variant using voltage-clamp recordings to study voltage-

352   dependent sodium currents. We examined the biophysical properties of the total sodium

353   current passed by DRG neurons transfected with either wild-type β2 or β2-Y69H

354   plasmids (Figure 4). The Y69H variant did not significantly alter the half-maximal voltage

355   of activation ($V_{1/2}$, -21.04 ± 1.40 mV, n = 18) for total sodium current compared to

356   neurons expressing the wild-type β2 subunits (-16.78 ± 2.18 mV, n = 15, Student's t-test

357   p = 0.10), although the point estimates showed an approximately 4.3 mV

358   hyperpolarization (Figure 4A). Similarly, the slope of the activation curves showed a

359   trend towards faster activation of sodium current in neurons expressing the Y69H

360   variant (6.37 ± 0.45 ms, n = 18) compared to wild-type (8.72 ± 1.20 ms, n = 15,

361   Student's t-test p = 0.059). In addition to activation, we also examined the effect of the

362   variant β2 subunit on sodium current inactivation (Figure 4A). Current traces were best

363   fit with a double Boltzmann equation and showed no difference in the $V_{1/2}$ of fast-

364   inactivation or the slope for the development of fast-inactivation between the Y69H

365   variant and the wild-type subunit (Table 1).

366   The Y69H variant caused no reduction in total sodium current use-dependent inhibition

367   (Figure 4B); after 1.5 seconds of 20 Hz pulses to -20 mV, neurons expressing the wild-

368   type human β2 subunit passed 76.12% of the current passed during the first stimulus,

369   which was comparable to neurons expressing the Y69H variant (74.85%). Likewise,

370   neurons expressing either construct showed a similar extent of channel recovery

371   (Figure 4C) and there was no change in the rate of recovery from fast-inactivation

372   between neurons expressing the wild-type and the Y69H variant (Figure 4D). Finally, we

373   assessed the rate of channel deactivation (Figure 4E) and found no change between

374   DRG neurons expressing the wild-type or Y69H variant β2 subunit.

17

*Tetrodotoxin-sensitive currents are upregulated by the β2-Y69H variant*

Given that there were no major biophysical changes conferred on sodium channels by the Y69H variant β2 subunit, we then examined sodium channel current density as the β-subunits are known to enhance channel trafficking to the plasma membrane(44, 66). DRG neurons were held at -100 mV and then pulsed to a range of potentials from -80 mV to + 30 mV for 100 ms in 5 mV increments and the current density was calculated at each step pulse. Neurons expressing the Y69H variant exhibited a significantly larger peak total sodium current density (-590.90 ± 46.21 pA/pF, n = 21) after a 100 ms pulse compared to DRG neurons expressing the wild-type β2 subunit (-359.11 ± 36.11 pA/pF, n = 17, Student's t-test p = 0.00052, Figure 5B). Additionally, as current density is derived from both the amplitude of the currents, as well as the capacitance of the neurons, we sought to confirm that there was no difference in the capacitance of the cells recorded. The capacitance of DRG neurons expressing the Y69H variant β2 subunit (22.91 ± 2.05 pF, n = 21) was similar to that of DRG neurons expressing the wild-type subunit (21.80 ± 2.42 pF, n = 17, Student's t-test p = 0.73).

We next sought to determine whether the upregulated sodium currents were TTX-S or TTX-R. The same voltage protocol as above was applied first in the absence of TTX, then after perfusion of 1 μM into the recording chamber. TTX-S current was calculated by reference series subtraction of TTX-R current from the total sodium current (Figure 5A). It is important to note that a voltage error greater than 5 mV was an exclusion criterion in the first set of experiments to minimize uncertainty in biophysical analysis of channel gating. In this second set of experiments, however, voltage error was not considered. This was a decision consciously made in order to avoid artificially limiting

18

398     any difference between the wild-type and Y69H-expressing neurons by excluding cells

399     with large currents. Crucially, voltage error would not affect the determination of sodium

400     current density. We were able to confirm again, in this second set of experiments, an

401     upregulation of total $Na_V$ current in DRG neurons expressing the Y69H variant

402     compared to DRG neurons expressing the wild-type variant (Y69H: -879.82 ± 261.94

403     pA/pF, n = 15; wild-type: -435.02 ± 69.42 pA/pF, n = 26; Student's t-test p = 0.048).

404     However, we did not detect a statistically significant difference in TTX-R current in cells

405     expressing the YH variant (-214.273 ± 44.81 pA/pF, n = 15) compared to DRG neurons

406     expressing the wild-type variant (-189.30 ± 32.47 pA/pF, n = 26, Student's t-test p =

407     0.65). By contrast, TTX-S current was more than doubled (Figure 5C) in DRG neurons

408     expressing the Y69H variant (-768.21 ± 234.99 pA/pF, n = 15), relative to TTX-S current

409     in DRG neurons expressing the wild-type β2 subunit (-330.23 ± 66.27 pA/pF, n = 26,

410     Student's t-test p = 0.032).

411     As DRG neurons express a multiple $Na_V$ channel isoforms, we sought to investigate the

412     Y69H variant in a heterologous system expressing only $Na_V1.7$, which is responsible for

413     the majority of TTX-S sodium currents in rat DRG neurons (67). Consistent with our

414     previous results in DRG neurons, we confirmed that the β2-Y69H variant does not alter

415     gating properties (Figure 5D) of $Na_V1.7$ channels stably expressed in HEK293 cells.

416     Specifically, the $V_{1/2}$ of activation of $Na_V1.7$ in HEK293 cells transfected with the wild-

417     type β2 subunit was -22.97 ± 1.58 mV (n = 16), which is comparable to those

418     transfected with the Y69H variant (-21.79 ± 1.32, n = 20, Student's t-test p = 0.57).

419     Similarly, we did not observe a difference in the $V_{1/2}$ of fast-inactivation of Nav1.7

420     channels in HEK293 cells transfected with the wild-type β2 subunit (-79.60 ± 1.54 mV, n

19

421   = 17), compared to those transfected with the Y69H variant (-79.73 ± 1.67 mV, n = 20,

422   Student's t-test p = 0.96). Interestingly, we observed no statistically significant difference

423   in the $Na_V1.7$ current density (Figure 5E) in these cells, either: current density of Nav1.7

424   in cells transfected with the wild-type β2 subunit (-572.67 ± 64.71 pA/pF, n = 31) was

425   not different from that in cells transfected with the Y69H variant (-686.57 ± 57.84 pA/pF,

426   n = 30, Student's t-test p = 0.20).

427

**Discussion**

428

429    While the contribution of β-subunits to sodium channel physiology and neuronal

430    excitability has long been studied, only recently have mutations in β-subunits, and

431    specifically the β2-subunit, been linked to human pain disorders. Here we document the

432    first case of idiopathic small-fiber neuropathy associated with a mutation in the *SCN2B*

433    gene. The expression of the β2-Y69H mutation in small-diameter DRG neurons

434    increases current density of TTX-S channels and renders these neurons hyperexcitable,

435    compared to the wild-type human β2 subunit (Figure 6). The expression of the β2-Y69H

436    subunit reduces the threshold to action potential firing, allowing for enhanced repetitive

437    action potential firing. Interestingly, the mechanism underlying this hyperexcitability is a

438    significant increase in TTX-S current density, rather than a major change in channel

439    gating properties. We also report that the co-expression of the β2-Y69H mutant with

440    Nav1.7 channels in HEK293 cells does not recapitulate the increased current density

441    that we observe in DRG neurons, suggesting a cell background-specific mechanism to

442    unmask the effect of this β2 subunit mutation.

443    The association of β subunits with the pore-forming α subunits has been shown to

444    regulate channel trafficking to the plasma membrane and gating properties (38). While

445    gain-of-function mutations in α subunits leading to neuropathic pain typically result in

446    biophysical shifts in voltage-dependences of activation or inactivation, or altered

447    recovery from inactivation, changes in channel current density alone are sufficient to

448    alter the excitability of DRG neurons (67); increasing the current density of $Na_V1.7$,

449    which accounts for approximately 70% of TTX-S current density in small-diameter DRG

450    neurons, both reduces the current threshold to action potential firing and enhances

21

451    repetitive action potential spiking(67). Vasylyev *et al.* used dynamic clamp to precisely

452    modulate the levels of $Na_V1.7$ current in small-diameter DRG neurons and showed that

453    increasing $Na_V1.7$ current density by 100% resulted in a near 30% reduction in current

454    threshold. While it would be interesting to compare our results directly to the results

455    from that study, it is not feasible for multiple reasons. Primarily, dynamic clamp injects

456    current using an equation modeling a specific ion channel, but the levels of the physical

457    channel are unchanged. Because we presumably had an increase in the number of

458    TTX-S channels at the cell surface, this may also have affected some change to

459    neuronal excitability, possibly by an interaction with one or more of their many channel

460    binding-partners(68). Additionally, Vasylyev et al. only modulated up to a 100% change

461    in $Na_V1.7$ levels, whereas we recorded an approximately 130% increase in TTX-S

462    current density, so a comparison would both require an extrapolation of their generated

463    fit, as well as an assumption that the Y69H variant increases $Na_V1.7$ current density

464    exclusively; indeed, we were unable to confirm in a heterologous expression system

465    that the Y69H variant upregulates $Na_V1.7$ currents. This may imply that the Y69H

466    variant upregulates other TTX-S $Na_V$ channels, such as $Na_V1.6$, which has also been

467    implicated in pain disorders(69) and shown to regulate sensory neuronal excitability(70).

468    Alternatively, $Na_V1.7$ may be a target of upregulation by the Y69H variant, but

469    differences in trafficking mechanisms between DRG neurons and HEK293 cells

470    obscured our ability to detect an effect in the latter system. Regardless, it remains clear

471    that the increase in TTX-S current density conferred by the β2-Y69H variant is

472    consistent with neuronal hyperexcitability.

22

473    Previously, we reported and characterized the first β-subunit mutation, an aspartate-to-

474    asparagine substitution at position 109 in the β2 subunit, in a patient with a pain

475    syndrome—specifically, diabetic neuropathy(59). Although the D109N variant was

476    discovered in a patient with underlying longstanding diabetes, functional validation of

477    that variant confirmed that it induces neuronal hyperexcitability. Although it is not clear

478    why pain and neuropathy in the patient described here were not manifested until the

479    fifth decade, the discovery of the Y69H β2 variant, which increased the current density

480    of the TTX-S currents, further strengthens a contributory link between the β2 subunit

481    and neuropathic pain as no other putative cause of pain was identified.

482    The purported mechanism of action of both the newly identified Y69H variant and the

483    previously identified D109N variant is concordant with established effects of the β2

484    subunit in DRG neurons. All β-subunits, with the exception of the β1b splice variant,

485    share the common structure of an extracellular immunoglobulin domain and a

486    transmembrane domain with a short cytoplasmic tail. The Y69H variant is located in the

487    extracellular immunoglobulin domain, which binds to Nav channels within the DII-S5-6

488    extracellular linker via a disulfide bond(39, 71) and enhances channel trafficking to the

489    plasma membrane. This is consistent with the known role of β2 subunits in small-

490    diameter DRG neurons; Lopez-Santiago *et al.* showed that β2-null mice DRG neurons

491    exhibited approximately a 50% reduction in TTX-S current and $Na_V1.7$ protein

492    levels(48).

493    The β2-Y69H variant was also identified in two patients with Brugada Syndrome, and,

494    when co-expressed with $Na_V1.5$ in HEK293 cells, resulted in a reduction in current

495    density(72). The data from this early study has only been available in a meeting

23

496    abstract, and is thus difficult to evaluate. By contrast, the present studies were done in

497    DRG neurons where a full complement of additional factors could be important for

498    uncovering the true effects of this mutation on the TTX-S channels. This data suggests

499    that the Y69H variant may exert its effect in an isoform-specific manner or in a cell

500    background-dependent manner.

501    Both the D109N and Y69H variants are located in the extracellular immunoglobulin

502    domain and both act on TTX-S but not TTX-R channels; however, the mechanism of

503    action for the Y69H variant is distinct from that of the D109N variant. This different

504    mode of action may be influenced by the position of the two residues in the folded

505    structure of the β2 subunit. The side chain of the Y69H variant points away from the $Na_V$

506    α subunit (Figure 1), whereas the D109N mutant residue is located in a spatially

507    separate and distinct location within the β2 subunit. We postulate that mutations in

508    residues at different loci in the β2 subunit may perturb their respective local structures

509    and interfere differentially with normal subunit functioning. For example, based on the

510    data presented in this manuscript, we postulate that mutations facing away from the

511    channel may be more likely to modulate channel trafficking to the cell surface or

512    enhance channel stability at the plasma membrane via interactions with other

513    membrane proteins or extracellular matrix proteins. On the other hand, mutations within

514    local environments closer to the α subunits of TTX-S channels may engage in

515    interactions with the channel itself and cause biophysical alterations in their gating

516    properties. These modes of action of mutations in the β2 subunit are difficult to predict

517    using static modeling even with the advances in determining structures of Nav subunits

518    at the atomic level because these structures do not capture the dynamic nature of

519    conformational changes of the channel within the plasma membrane during gating

520    steps.

521    There is a paucity of publications of functional characterization of β2 mutations

522    associated with excitability disorders, highlighting the need to fill in this knowledge gap

523    to better understand the contribution of these subunits to normal neuronal physiology

524    and to the effects of mutations of this subunit in the pathophysiology of human pain

525    disorders. The data that we present here, together with our previous publication of a β2

526    mutation in a patient with painful diabetic neuropathy(59) strengthens the evidence for a

527    role of these subunits in the pathophysiology of human pain disorders.

528

25

## References

1.	**Faber CG, Hoeijmakers JG, Ahn HS, Cheng X, Han C, Choi JS, Estacion M, Lauria G, Vanhoutte EK, Gerrits MM, Dib-Hajj S, Drenth JP, Waxman SG, and Merkies IS**. Gain of function Nav1.7 mutations in idiopathic small fiber neuropathy. *Ann Neurol* 71: 26-39, 2012.

2.	**Sopacua M, Hoeijmakers JGJ, Merkies ISJ, Lauria G, Waxman SG, and Faber CG**. Small-fiber neuropathy: Expanding the clinical pain universe. *J Peripher Nerv Syst* 24: 19-33, 2019.

3.	**Tesfaye S, Boulton AJ, Dyck PJ, Freeman R, Horowitz M, Kempler P, Lauria G, Malik RA, Spallone V, Vinik A, Bernardi L, and Valensi P**. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. *Diabetes Care* 33: 2285-2293, 2010.

4.	**Han Y, and Smith MT**. Pathobiology of cancer chemotherapy-induced peripheral neuropathy (CIPN). *Front Pharmacol* 4: 156, 2013.

5.	**Devigili G, Rinaldo S, Lombardi R, Cazzato D, Marchi M, Salvi E, Eleopra R, and Lauria G**. Diagnostic criteria for small fibre neuropathy in clinical practice and research. *Brain* 142: 3728-3736, 2019.

6.	**Bakkers M, Faber CG, Hoeijmakers JG, Lauria G, and Merkies IS**. Small fibers, large impact: quality of life in small-fiber neuropathy. *Muscle Nerve* 49: 329-336, 2014.

7.	**Schaefer C, Mann R, Sadosky A, Daniel S, Parsons B, Nalamachu S, Stacey BR, Tuchman M, Anschel A, and Nieshoff E**. Health status, function, productivity, and costs among individuals with idiopathic painful peripheral neuropathy with small fiber involvement in the United States: results from a retrospective chart review and cross-sectional survey. *J Med Econ* 17: 394-407, 2014.

8.	**Nishikawa N, and Nomoto M**. Management of neuropathic pain. *J Gen Fam Med* 18: 56-60, 2017.

9.	**Finnerup NB, Attal N, Haroutounian S, McNicol E, Baron R, Dworkin RH, Gilron I, Haanpaa M, Hansson P, Jensen TS, Kamerman PR, Lund K, Moore A, Raja SN, Rice AS, Rowbotham M, Sena E, Siddall P, Smith BH, and Wallace M**. Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. *Lancet Neurol* 14: 162-173, 2015.

10.	**de Greef BTA, Hoeijmakers JGJ, Gorissen-Brouwers CML, Geerts M, Faber CG, and Merkies ISJ**. Associated conditions in small fiber neuropathy - a large cohort study and review of the literature. *Eur J Neurol* 25: 348-355, 2018.

11.	**Brouwer BA, Merkies IS, Gerrits MM, Waxman SG, Hoeijmakers JG, and Faber CG**. Painful neuropathies: the emerging role of sodium channelopathies. *J Peripher Nerv Syst* 19: 53-65, 2014.

12.	**Lehmann HC, Wunderlich G, Fink GR, and Sommer C**. Diagnosis of peripheral neuropathy. *Neurol Res Pract* 2: 20, 2020.

13.	**Huang J, Yang Y, Zhao P, Gerrits MM, Hoeijmakers JG, Bekelaar K, Merkies IS, Faber CG, Dib-Hajj SD, and Waxman SG**. Small-Fiber Neuropathy Nav1.8 Mutation Shifts Activation to Hyperpolarized Potentials and Increases Excitability of Dorsal Root Ganglion Neurons. *J Neurosci* 33: 14087-14097, 2013.

14.	**Faber CG, Lauria G, Merkies IS, Cheng X, Han C, Ahn HS, Persson AK, Hoeijmakers JG, Gerrits MM, Pierro T, Lombardi R, Kapetis D, Dib-Hajj SD, and Waxman SG**. Gain-of-function Nav1.8 mutations in painful neuropathy. *Proc Natl Acad Sci U S A* 109: 19444-19449, 2012b.

15.	**Huang J, Han C, Estacion M, Vasylyev D, Hoeijmakers JG, Gerrits MM, Tyrrell L, Lauria G, Faber CG, Dib-Hajj SD, Merkies IS, Waxman SG, and Group PS**. Gain-of-function mutations in sodium channel Na(v)1.9 in painful neuropathy. *Brain* 137: 1627-1642, 2014.

16.	**Eijkenboom I, Sopacua M, Hoeijmakers JGJ, de Greef BTA, Lindsey P, Almomani R, Marchi M, Vanoevelen J, Smeets HJM, Waxman SG, Lauria G, Merkies ISJ, Faber CG, and Gerrits MM**. Yield of peripheral sodium channels gene screening in pure small fibre neuropathy. *J Neurol Neurosurg Psychiatry* 90: 342-352, 2019.

26

575     17.     **Vlckova-Moravcova E, Bednarik J, Belobradkova J, and Sommer C**. Small-fibre involvement in
576 diabetic patients with neuropathic foot pain. *Diabet Med* 25: 692-699, 2008.
577     18.     **Brewer JR, Morrison G, Dolan ME, and Fleming GF**. Chemotherapy-induced peripheral
578 neuropathy: Current status and progress. *Gynecol Oncol* 140: 176-183, 2016.
579     19.     **Bednarik J, Vlckova-Moravcova E, Bursova S, Belobradkova J, Dusek L, and Sommer C**. Etiology
580 of small-fiber neuropathy. *J Peripher Nerv Syst* 14: 177-183, 2009.
581     20.     **de Greef BTA, Hoeijmakers JGJ, Geerts M, Oakes M, Church TJE, Waxman SG, Dib-Hajj SD,**
582 **Faber CG, and Merkies ISJ**. Lacosamide in patients with Nav1.7 mutations-related small fibre
583 neuropathy: a randomized controlled trial. *Brain* 142: 263-275, 2019.
584     21.     **Waxman SG**. The neuron as a dynamic electrogenic machine: modulation of sodium-channel
585 expression as a basis for functional plasticity in neurons. *Philos Trans R Soc Lond B Biol Sci* 355: 199-213,
586 2000.
587     22.     **Hodgkin AL, and Huxley AF**. A quantitative description of membrane current and its application
588 to conduction and excitation in nerve. *J Physiol* 117: 500-544, 1952.
589     23.     **Catterall WA, Lenaeus MJ, and Gamal El-Din TM**. Structure and Pharmacology of Voltage-Gated
590 Sodium and Calcium Channels. *Annu Rev Pharmacol Toxicol* 60: 133-154, 2020.
591     24.     **Yu FH, and Catterall WA**. Overview of the voltage-gated sodium channel family. *Genome Biol* 4:
592 207, 2003.
593     25.     **Dib-Hajj SD, Yang Y, Black JA, and Waxman SG**. The Na(V)1.7 sodium channel: from molecule to
594 man. *Nat Rev Neurosci* 14: 49-62, 2013.
595     26.     **Han C, Huang J, and Waxman SG**. Sodium channel Nav1.8: Emerging links to human disease.
596 *Neurology* 86: 473-483, 2016.
597     27.     **Bennett DL, Clark AJ, Huang J, Waxman SG, and Dib-Hajj SD**. The Role of Voltage-Gated Sodium
598 Channels in Pain Signaling. *Physiol Rev* 99: 1079-1151, 2019.
599     28.     **Yang Y, Wang Y, Li S, Xu Z, Li H, Ma L, Fan J, Bu D, Liu B, Fan Z, Wu G, Jin J, Ding B, Zhu X, and**
600 **Shen Y**. Mutations in SCN9A, encoding a sodium channel alpha subunit, in patients with primary
601 erythermalgia. *J Med Genet* 41: 171-174, 2004.
602     29.     **Cummins TR, Dib-Hajj SD, and Waxman SG**. Electrophysiological properties of mutant Nav1.7
603 sodium channels in a painful inherited neuropathy. *J Neurosci* 24: 8232-8236, 2004.
604     30.     **Dib-Hajj SD, Rush AM, Cummins TR, Hisama FM, Novella S, Tyrrell L, Marshall L, and Waxman**
605 **SG**. Gain-of-function mutation in Nav1.7 in familial erythromelalgia induces bursting of sensory neurons.
606 *Brain* 128: 1847-1854, 2005.
607     31.     **Han C, Rush AM, Dib-Hajj SD, Li S, Xu Z, Wang Y, Tyrrell L, Wang X, Yang Y, and Waxman SG**.
608 Sporadic onset of erythermalgia: a gain-of-function mutation in Nav1.7. *Ann Neurol* 59: 553-558, 2006.
609     32.     **Lampert A, Dib-Hajj SD, Tyrrell L, and Waxman SG**. Size matters: Erythromelalgia mutation
610 S241T in Nav1.7 alters channel gating. *J Biol Chem* 281: 36029-36035, 2006.
611     33.     **Cox JJ, Reimann F, Nicholas AK, Thornton G, Roberts E, Springell K, Karbani G, Jafri H, Mannan**
612 **J, Raashid Y, Al-Gazali L, Hamamy H, Valente EM, Gorman S, Williams R, McHale DP, Wood JN, Gribble**
613 **FM, and Woods CG**. An SCN9A channelopathy causes congenital inability to experience pain. *Nature*
614 444: 894-898, 2006.
615     34.     **Goldberg YP, MacFarlane J, MacDonald ML, Thompson J, Dube MP, Mattice M, Fraser R,**
616 **Young C, Hossain S, Pape T, Payne B, Radomski C, Donaldson G, Ives E, Cox J, Younghusband HB, Green**
617 **R, Duff A, Boltshauser E, Grinspan GA, Dimon JH, Sibley BG, Andria G, Toscano E, Kerdraon J, Bowsher**
618 **D, Pimstone SN, Samuels ME, Sherrington R, and Hayden MR**. Loss-of-function mutations in the Nav1.7
619 gene underlie congenital indifference to pain in multiple human populations. *Clin Genet* 71: 311-319,
620 2007.

621    35.    **Ahmad S, Dahllund L, Eriksson AB, Hellgren D, Karlsson U, Lund PE, Meijer IA, Meury L, Mills T,**
622    **Moody A, Morinville A, Morten J, O'Donnell D, Raynoschek C, Salter H, Rouleau GA, and Krupp JJ**. A
623    stop codon mutation in SCN9A causes lack of pain sensation. *Hum Mol Genet* 16: 2114-2121, 2007.
624    36.    **Han C, Vasylyev D, Macala LJ, Gerrits MM, Hoeijmakers JG, Bekelaar KJ, Dib-Hajj SD, Faber CG,**
625    **Merkies IS, and Waxman SG**. The G1662S NaV1.8 mutation in small fibre neuropathy: impaired
626    inactivation underlying DRG neuron hyperexcitability. *J Neurol Neurosurg Psychiatry* 85: 499-505, 2014.
627    37.    **Chahine M, and O'Leary ME**. Regulatory Role of Voltage-Gated Na Channel beta Subunits in
628    Sensory Neurons. *Front Pharmacol* 2: 70, 2011.
629    38.    **Bouza AA, and Isom LL**. Voltage-Gated Sodium Channel β Subunits and Their Related Diseases.
630    *Handb Exp Pharmacol* 246: 423-450, 2018.
631    39.    **Shen H, Liu D, Wu K, Lei J, and Yan N**. Structures of human Na(v)1.7 channel in complex with
632    auxiliary subunits and animal toxins. *Science* 363: 1303-1308, 2019.
633    40.    **Mantegazza M, and Catterall WA**. Voltage-Gated Na(+) Channels: Structure, Function, and
634    Pathophysiology. In: *Jasper's Basic Mechanisms of the Epilepsies*, edited by Noebels JL, Avoli M,
635    Rogawski MA, Olsen RW, and Delgado-Escueta AV. Bethesda MD: © 2012, Michael A Rogawski, Antonio
636    V Delgado-Escueta, Jeffrey L Noebels, Massimo Avoli and Richard W Olsen., 2012.
637    41.    **Sokolov MV, Henrich-Noack P, Raynoschek C, Franzén B, Larsson O, Main M, and Dabrowski**
638    **M**. Co-expression of β Subunits with the Voltage-Gated Sodium Channel Na(V)1.7: the Importance of
639    Subunit Association and Phosphorylation and Their Effects on Channel Pharmacology and Biophysics. *J*
640    *Mol Neurosci* 65: 154-166, 2018.
641    42.    **Cusdin FS, Nietlispach D, Maman J, Dale TJ, Powell AJ, Clare JJ, and Jackson AP**. The sodium
642    channel {beta}3-subunit induces multiphasic gating in NaV1.3 and affects fast inactivation via distinct
643    intracellular regions. *J Biol Chem* 285: 33404-33412, 2010.
644    43.    **Johnson D, Montpetit ML, Stocker PJ, and Bennett ES**. The sialic acid component of the beta1
645    subunit modulates voltage-gated sodium channel function. *J Biol Chem* 279: 44303-44310, 2004.
646    44.    **Isom LL, De Jongh KS, Patton DE, Reber BF, Offord J, Charbonneau H, Walsh K, Goldin AL, and**
647    **Catterall WA**. Primary structure and functional expression of the beta 1 subunit of the rat brain sodium
648    channel. *Science* 256: 839-842, 1992.
649    45.    **Brackenbury WJ, Calhoun JD, Chen C, Miyazaki H, Nukina N, Oyama F, Ranscht B, and Isom LL**.
650    Functional reciprocity between Na+ channel Nav1.6 and beta1 subunits in the coordinated regulation of
651    excitability and neurite outgrowth. *Proc Natl Acad Sci U S A* 107: 2283-2288, 2010.
652    46.    **Laedermann CJ, Syam N, Pertin M, Decosterd I, and Abriel H**. β1- and β3- voltage-gated sodium
653    channel subunits modulate cell surface expression and glycosylation of Nav1.7 in HEK293 cells. *Front Cell*
654    *Neurosci* 7: 137, 2013.
655    47.    **Cummins TR, Aglieco F, Renganathan M, Herzog RI, Dib-Hajj SD, and Waxman SG**. Nav1.3
656    sodium channels: rapid repriming and slow closed-state inactivation display quantitative differences
657    after expression in a mammalian cell line and in spinal sensory neurons. *J Neurosci* 21: 5952-5961, 2001.
658    48.    **Lopez-Santiago LF, Pertin M, Morisod X, Chen C, Hong S, Wiley J, Decosterd I, and Isom LL**.
659    Sodium channel beta2 subunits regulate tetrodotoxin-sensitive sodium channels in small dorsal root
660    ganglion neurons and modulate the response to pain. *J Neurosci* 26: 7984-7994, 2006.
661    49.    **Chen C, Bharucha V, Chen Y, Westenbroek RE, Brown A, Malhotra JD, Jones D, Avery C,**
662    **Gillespie PJ, 3rd, Kazen-Gillespie KA, Kazarinova-Noyes K, Shrager P, Saunders TL, Macdonald RL,**
663    **Ransom BR, Scheuer T, Catterall WA, and Isom LL**. Reduced sodium channel density, altered voltage
664    dependence of inactivation, and increased susceptibility to seizures in mice lacking sodium channel beta
665    2-subunits. *Proc Natl Acad Sci U S A* 99: 17072-17077, 2002.
666    50.    **Shimizu H, Tosaki A, Ohsawa N, Ishizuka-Katsura Y, Shoji S, Miyazaki H, Oyama F, Terada T,**
667    **Shirouzu M, Sekine SI, Nukina N, and Yokoyama S**. Parallel homodimer structures of the extracellular

28

domains of the voltage-gated sodium channel beta4 subunit explain its role in cell-cell adhesion. *J Biol Chem* 292: 13428-13440, 2017.

51.     **Xiong H, Yang Q, Zhang X, Wang P, Chen F, Liu Y, Wang P, Zhao Y, Li S, Huang Y, Chen S, Wang X, Zhang H, Yu D, Tan C, Fang C, Huang Y, Wu G, Wu Y, Cheng X, Liao Y, Zhang R, Yang Y, Ke T, Ren X, Li H, Tu X, Xia Y, Xu C, Chen Q, and Wang QK**. Significant association of rare variant p.Gly8Ser in cardiac sodium channel β4-subunit SCN4B with atrial fibrillation. *Ann Hum Genet* 83: 239-248, 2019.

52.     **Riuró H, Beltran-Alvarez P, Tarradas A, Selga E, Campuzano O, Vergés M, Pagans S, Iglesias A, Brugada J, Brugada P, Vázquez FM, Pérez GJ, Scornik FS, and Brugada R**. A missense mutation in the sodium channel β2 subunit reveals SCN2B as a new candidate gene for Brugada syndrome. *Hum Mutat* 34: 961-966, 2013.

53.     **Wallace RH, Wang DW, Singh R, Scheffer IE, George AL, Jr., Phillips HA, Saar K, Reis A, Johnson EW, Sutherland GR, Berkovic SF, and Mulley JC**. Febrile seizures and generalized epilepsy associated with a mutation in the Na+-channel beta1 subunit gene SCN1B. *Nat Genet* 19: 366-370, 1998.

54.     **Meadows LS, Malhotra J, Loukas A, Thyagarajan V, Kazen-Gillespie KA, Koopman MC, Kriegler S, Isom LL, and Ragsdale DS**. Functional and biochemical analysis of a sodium channel beta1 subunit mutation responsible for generalized epilepsy with febrile seizures plus type 1. *J Neurosci* 22: 10699-10709, 2002.

55.     **Audenaert D, Claes L, Ceulemans B, Löfgren A, Van Broeckhoven C, and De Jonghe P**. A deletion in SCN1B is associated with febrile seizures and early-onset absence epilepsy. *Neurology* 61: 854-856, 2003.

56.     **Patino GA, Claes LR, Lopez-Santiago LF, Slat EA, Dondeti RS, Chen C, O'Malley HA, Gray CB, Miyazaki H, Nukina N, Oyama F, De Jonghe P, and Isom LL**. A functional null mutation of SCN1B in a patient with Dravet syndrome. *J Neurosci* 29: 10764-10778, 2009.

57.     **Pertin M, Ji RR, Berta T, Powell AJ, Karchewski L, Tate SN, Isom LL, Woolf CJ, Gilliard N, Spahn DR, and Decosterd I**. Upregulation of the voltage-gated sodium channel beta2 subunit in neuropathic pain models: characterization of expression in injured and non-injured primary sensory neurons. *J Neurosci* 25: 10970-10980, 2005.

58.     **Xie W, Zhang J, Strong JA, and Zhang JM**. Role of Na(V)1.6 and Na(V)β4 Sodium Channel Subunits in a Rat Model of Low Back Pain Induced by Compression of the Dorsal Root Ganglia. *Neuroscience* 402: 51-65, 2019.

59.     **Alsaloum M, Estacion M, Almomani R, Gerrits MM, Bönhof GJ, Ziegler D, Malik R, Ferdousi M, Lauria G, Merkies IS, Faber CG, Dib-Hajj S, and Waxman SG**. A gain-of-function sodium channel β2-subunit mutation in painful diabetic neuropathy. *Mol Pain* 15: 1744806919849802, 2019.

60.     **Almomani R, Marchi M, Sopacua M, Lindsey P, Salvi E, Koning B, Santoro S, Magri S, Smeets HJM, Martinelli Boneschi F, Malik RR, Ziegler D, Hoeijmakers JGJ, Bonhof G, Dib-Hajj S, Waxman SG, Merkies ISJ, Lauria G, Faber CG, Gerrits MM, and on behalf on the PSG**. Evaluation of molecular inversion probe versus TruSeq(R) custom methods for targeted next-generation sequencing. *PLoS One* 15: e0238467, 2020.

61.     **Lossin C, Wang DW, Rhodes TH, Vanoye CG, and George AL, Jr.** Molecular basis of an inherited epilepsy. *Neuron* 34: 877-884, 2002.

62.     **Dib-Hajj SD, Choi JS, Macala LJ, Tyrrell L, Black JA, Cummins TR, and Waxman SG**. Transfection of rat or mouse neurons by biolistics or electroporation. *Nat Protoc* 4: 1118-1126, 2009.

63.     **Jo S, Kim T, Iyer VG, and Im W**. CHARMM-GUI: a web-based graphical user interface for CHARMM. *J Comput Chem* 29: 1859-1865, 2008.

64.     **Karczewski KJ, Francioli LC, Tiao G, Cummings BB, Alföldi J, Wang Q, Collins RL, Laricchia KM, Ganna A, Birnbaum DP, Gauthier LD, Brand H, Solomonson M, Watts NA, Rhodes D, Singer-Berk M, England EM, Seaby EG, Kosmicki JA, Walters RK, Tashman K, Farjoun Y, Banks E, Poterba T, Wang A, Seed C, Whiffin N, Chong JX, Samocha KE, Pierce-Hoffman E, Zappala Z, O'Donnell-Luria AH, Minikel**

29

EV, Weisburd B, Lek M, Ware JS, Vittal C, Armean IM, Bergelson L, Cibulskis K, Connolly KM, Covarrubias M, Donnelly S, Ferriera S, Gabriel S, Gentry J, Gupta N, Jeandet T, Kaplan D, Llanwarne C, Munshi R, Novod S, Petrillo N, Roazen D, Ruano-Rubio V, Saltzman A, Schleicher M, Soto J, Tibbetts K, Tolonen C, Wade G, Talkowski ME, Aguilar Salinas CA, Ahmad T, Albert CM, Ardissino D, Atzmon G, Barnard J, Beaugerie L, Benjamin EJ, Boehnke M, Bonnycastle LL, Bottinger EP, Bowden DW, Bown MJ, Chambers JC, Chan JC, Chasman D, Cho J, Chung MK, Cohen B, Correa A, Dabelea D, Daly MJ, Darbar D, Duggirala R, Dupuis J, Ellinor PT, Elosua R, Erdmann J, Esko T, Färkkilä M, Florez J, Franke A, Getz G, Glaser B, Glatt SJ, Goldstein D, Gonzalez C, Group L, Haiman C, Hanis C, Harms M, Hiltunen M, Holi MM, Hultman CM, Kallela M, Kaprio J, Kathiresan S, Kim B-J, Kim YJ, Kirov G, Kooner J, Koskinen S, Krumholz HM, Kugathasan S, Kwak SH, Laakso M, Lehtimäki T, Loos RJF, Lubitz SA, Ma RCW, MacArthur DG, Marrugat J, Mattila KM, McCarroll S, McCarthy MI, McGovern D, McPherson R, Meigs JB, Melander O, Metspalu A, Neale BM, Nilsson PM, O'Donovan MC, Ongur D, Orozco L, Owen MJ, Palmer CNA, Palotie A, Park KS, Pato C, Pulver AE, Rahman N, Remes AM, Rioux JD, Ripatti S, Roden DM, Saleheen D, Salomaa V, Samani NJ, Scharf J, Schunkert H, Shoemaker MB, Sklar P, Soininen H, Sokol H, Spector T, Sullivan PF, Suvisaari J, Tai ES, Teo YY, Tiinamaija T, Tsuang M, Turner D, Tusie-Luna T, Vartiainen E, Ware JS, Watkins H, Weersma RK, Wessman M, Wilson JG, Xavier RJ, Neale BM, Daly MJ, MacArthur DG, and Genome Aggregation Database C. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature* 581: 434-443, 2020.

65.     Olesen MS, Andreasen L, Jabbari J, Refsgaard L, Haunsø S, Olesen SP, Nielsen JB, Schmitt N, and Svendsen JH. Very early-onset lone atrial fibrillation patients have a high prevalence of rare variants in genes previously associated with atrial fibrillation. *Heart Rhythm* 11: 246-251, 2014.

66.     Isom LL, Ragsdale DS, De Jongh KS, Westenbroek RE, Reber BF, Scheuer T, and Catterall WA. Structure and function of the beta 2 subunit of brain sodium channels, a transmembrane glycoprotein with a CAM motif. *Cell* 83: 433-442, 1995.

67.     Vasylyev DV, Han C, Zhao P, Dib-Hajj S, and Waxman SG. Dynamic-clamp analysis of wild-type human Nav1.7 and erythromelalgia mutant channel L858H. *J Neurophysiol* 111: 1429-1443, 2014.

68.     Kanellopoulos AH, Koenig J, Huang H, Pyrski M, Millet Q, Lolignier S, Morohashi T, Gossage SJ, Jay M, Linley JE, Baskozos G, Kessler BM, Cox JJ, Dolphin AC, Zufall F, Wood JN, and Zhao J. Mapping protein interactions of sodium channel NaV1.7 using epitope-tagged gene-targeted mice. *EMBO J* 37: 427-445, 2018.

69.     Tanaka BS, Zhao P, Dib-Hajj FB, Morisset V, Tate S, Waxman SG, and Dib-Hajj SD. A gain-of-function mutation in Nav1.6 in a case of trigeminal neuralgia. *Mol Med* 22: 338-348, 2016.

70.     Israel MR, Tanaka BS, Castro J, Thongyoo P, Robinson SD, Zhao P, Deuis JR, Craik DJ, Durek T, Brierley SM, Waxman SG, Dib-Hajj SD, and Vetter I. Na(V) 1.6 regulates excitability of mechanosensitive sensory neurons. *J Physiol* 597: 3751-3768, 2019.

71.     Chen C, Calhoun JD, Zhang Y, Lopez-Santiago L, Zhou N, Davis TH, Salzer JL, and Isom LL. Identification of the Cysteine Residue Responsible for Disulfide Linkage of Na+ Channel α and β2 Subunits*. *Journal of Biological Chemistry* 287: 39061-39069, 2012.

72.     Hu D, Barajas-Martinez H, Medeiros-Domingo A, Crotti L, Tester D, Veltmann C, Schimpf R, Pfeiffer R, Dezi F, Liu Y, Burashnikov E, Guidicessi JR, Ye D, Wolpert C, Borggrefe M, Schwartz P, Ackerman MJ, and Antzelevitch C. Abstract 16521: Novel Mutations in the Sodium Channel 2 Subunit Gene (SCN2B) Associated with Brugada Syndrome and Atrial Fibrillation. *Circulation* 126: A16521, 2012.

761  **Table 1.** Fast-inactivation properties in DRG neurons expressing either the wild-type or
762  Y69H β2 subunit.

|  | Wild-type (n = 14) | Y69H (n = 12) | Student's t-test p-value |
|---|---|---|---|
| TTX-R $V_{1/2}$ | -45.10 ± 1.81 | -43.61 ± 2.14 | 0.60 |
| TTX-R slope | 6.18 ± 0.32 | 6.035 ± 0.83 | 0.86 |
| TTX-S $V_{1/2}$ | -75.48 ± 1.76 | -79.22 ± 1.87 | 0.16 |
| TTX-S slope | 5.31 ± 0.59 | 5.89 ± 0.78 | 0.55 |

763

## Figure Legends

765  **Figure 1.** Molecular modeling of the β2-Y69H variant. The immunoglobulin domain of

766  the human β2 subunit (cyan) is linked to the $Na_V1.7$ channel α subunit (green) via a

767  disulfide bond with the cystine at position 55 (C55, yellow). The sidechain of the tyrosine

768  at position 69 (Y69, magenta) is shown, facing away from the α subunit. The star in the

769  cartoon diagram of the β2 subunit (top right) indicates the relative position of the

770  mutation. The sidechain of the aspartic acid at position 109 (D109, orange) is also

771  shown. The α subunit is embedded in a lipid bilayer membrane (red, white, and green

772  spheres). A small portion of the β1 subunit (red, top left) may be seen, as well.

773  **Figure 2.** The Y69H variant does not alter resting membrane potential of DRG neurons.

774  **(A)** Comparison of resting membrane potentials for DRG neurons expressing either the

775  wild-type human β2 subunit (monochrome) or the Y69H variant (orange) illustrates no

776  change in neuronal RMP. **(B)** A numerically larger, but not statistically significant,

777  proportion of DRG neurons expressing the Y69H variant fire spontaneous action

778  potentials at rest than DRG neurons expressing the wild-type human β2 subunit.

779  **Figure 3.** The Y69H variant reduces the threshold of action potential firing and

780  enhances repetitive spiking in DRG neurons. **(A)** Representative traces depicting the

781  action potential response of DRG neurons expressing the wild-type (black) or Y69H

31

782   variant (orange) human β2 subunit during stimulation with current injections of 200 ms

783   duration. The 0 mV membrane potential is indicated by the dashed line. **(B)** DRG

784   neurons expressing the Y69H variant displayed a significantly lower threshold to action

785   potential firing than did neurons expressing the wild-type β2 subunit. **(C)** When neurons

786   were stimulated with a 1000 ms pulse at the threshold amplitude as determined by a

787   200 ms injection in **(B)**, DRG neurons expressing the Y69H variant fired significantly

788   more action potentials than those expressing the wild-type β2 subunit. **(D)**

789   Representative traces depicting repetitive action potential firing of DRG neurons

790   expressing the Y69H variant (orange), compared to those expressing the wild-type β2

791   subunit (black). **(E)** Neurons expressing the Y69H variant (orange) fired significantly

792   more action potentials during a 1000 ms stimulus of graded amplitude than did neurons

793   expressing the wild-type β2 subunit (monochrome).

794   **Figure 4**. The Y69H variant does not alter total $Na_V$ channel gating properties. **(A)** The

795   voltage-dependence of channel inactivation (left; diamonds) are not statistically different

796   between DRG neurons expressing the wild-type and Y69H variant subunits.

797   Additionally, the voltage-dependence of activation (right, circles) is also not statistically

798   different. **(B)** There is no difference in the use-dependent inhibition of sodium channels

799   in DRG neurons expressing either the wild-type or the Y69H β2 subunit. **(C)** The extent

800   of sodium channel recovery from inactivation at voltages between -70 and -120 mV is

801   comparable for DRG neurons expressing either the wild-type or the variant β2 subunits.

802   **(D)** The rate of recovery from channel inactivation is also comparable in both groups.

803   **(E)** There is no difference in the kinetics of entry into deactivation between DRG
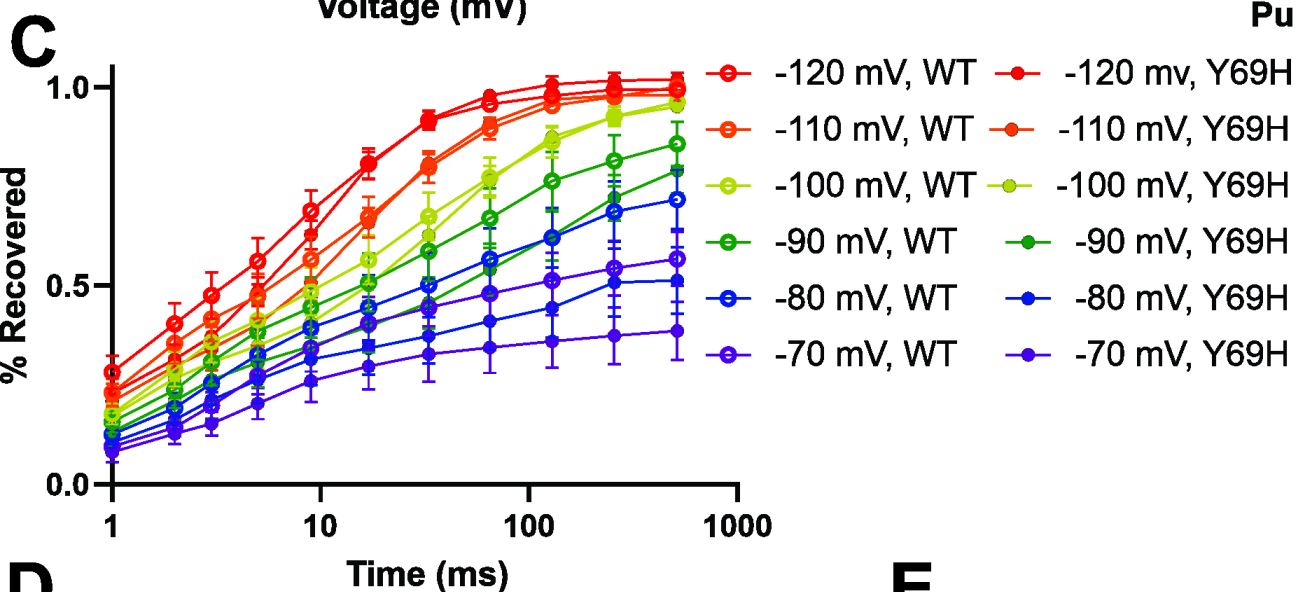
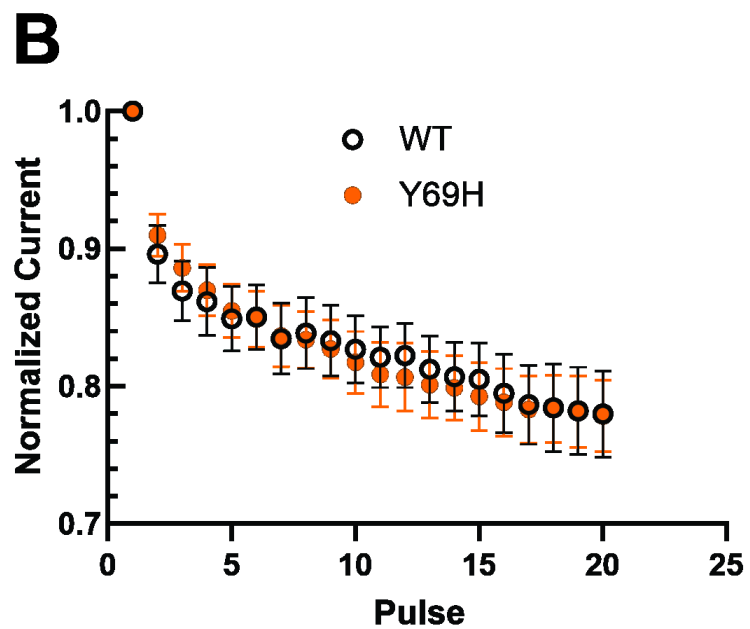804   neurons expressing the wild-type or Y69H variant.

32
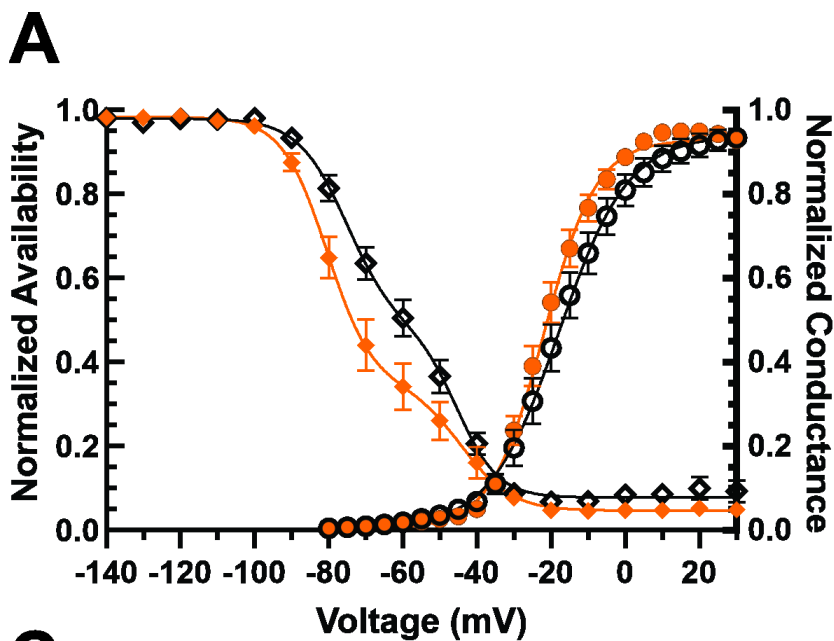
**Figure 5**. TTX-S current is upregulated in DRG neurons expressing Y69H β2 subunits.

**(A)** Representative traces illustrating reference series subtraction to determine TTX-S

current (right) from the difference between total sodium current (left) and TTX-R current

(middle) recorded in 1μM TTX. Example traces are shown for sodium currents in both

neurons expressing the wild-type β2 variant (top) and the Y69H variant (bottom,

orange). **(B)** There is a statistically significant increase in total sodium current passed by

DRG neurons expressing the Y69H variant compared to DRG neurons expressing the

wild-type β2 subunit. **(C)** TTX-S sodium currents in DRG neurons are upregulated in the

presence of the Y69H variant (orange), relative to the wild-type (monochrome) β2

subunit. However, there is no change in TTX-R sodium current. **(D)** The voltage-

dependence of $Na_V1.7$ channel inactivation (left; diamonds) is not statistically different

between HEK293 cells expressing the wild-type β2 (monochromatic) and the Y69H

variant (orange) subunits. Additionally, the voltage-dependence of activation (right,

circles) is also not statistically different. **(E)** The Y69H variant does not significantly

upregulate.7 current density in HEK293 cells stably expressing $Na_V1.7$ channels when
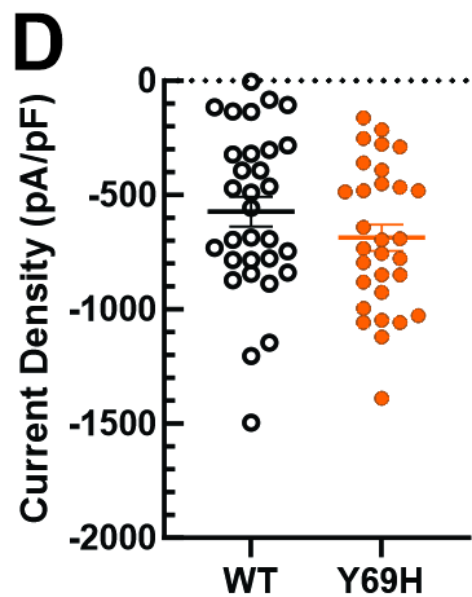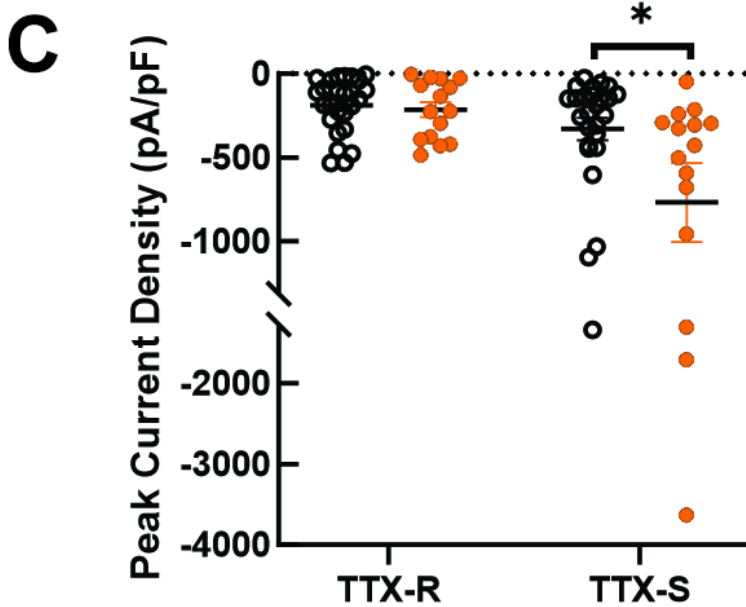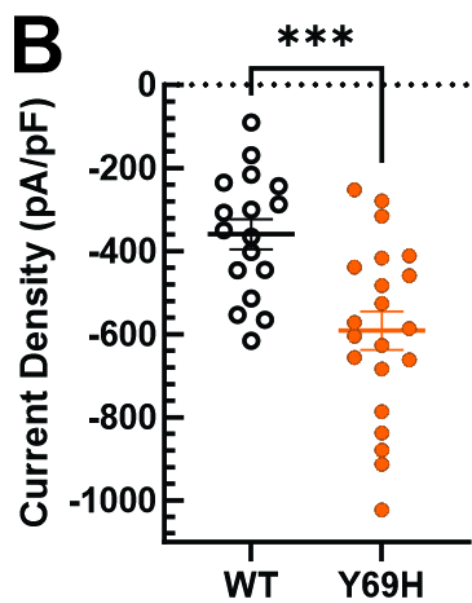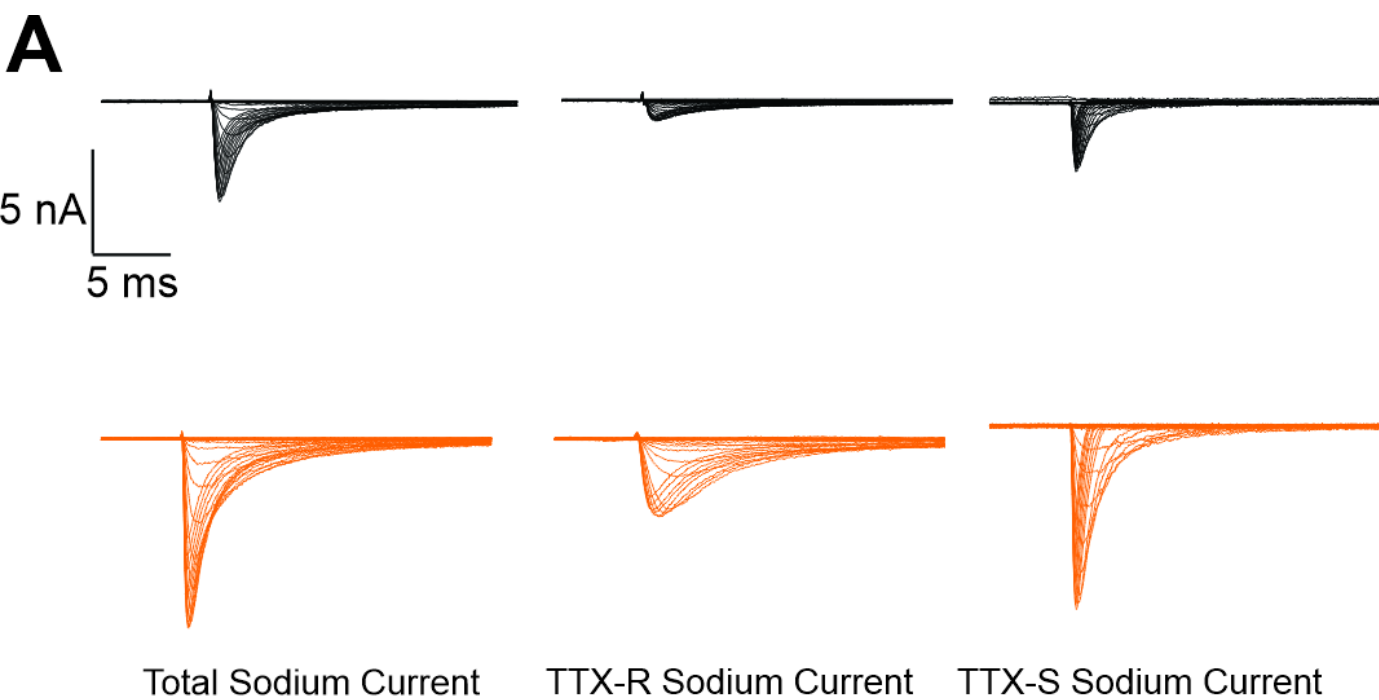
compared to the wild-type β2 subunit.

**Figure 6**. Schematic illustrating the proposed mechanism of action of the β2-Y69H

(right) on small-diameter DRG neurons. Compared to the wild-type β2 subunit (left),

neurons expressing the Y69H variant are hyperexcitable (top traces) and display a

larger TTX-S sodium current density (bottom traces), suggesting increased trafficking of
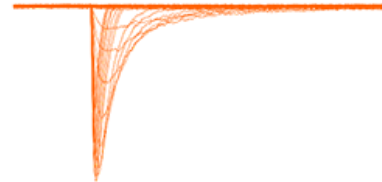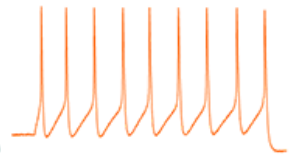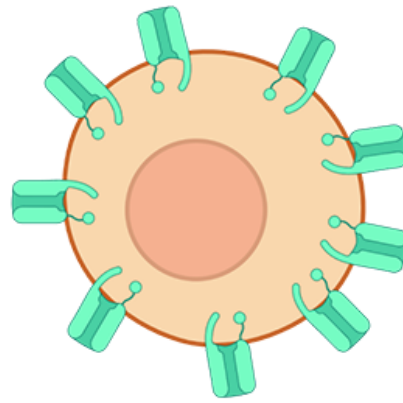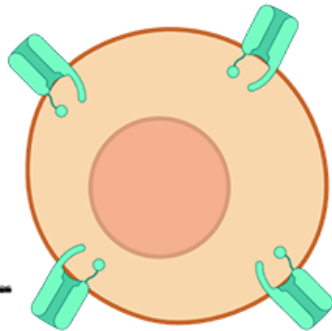
sodium channels to the neuronal membrane.

33

**A**

**B**

WT

3 cells

11 cells

■ ■ Spontaneous
▢ Evoked

Y69H

10 cells

11 cells

β2-Y69

β2-Y69H

β2-Y69

β2-Y69H