On the role of Nav1.7 sodium channels in chronic pain: an experimental and computational study

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Alberto Capurro[1*], Jack Thornton[1], Bruno Cessac[2], Lyle Armstrong[1], Evelyne Sernagor[1]

1. Biosciences Institute, Faculty of Medical Sciences, Newcastle University, UK.

2. Université Côte d'Azur, Inria, Biovision team, France.

Short title:

Chronic pain and Nav1.7 gating in human nociceptive neurons

* Corresponding author

E-mail: alberto.capurro@newcastle.ac.uk

1   **Abstract**

2   Chronic pain is a global healthcare problem with a huge societal impact. Its management remains

3   generally unsatisfactory, with no single treatment clinically approved in most cases. In this study we

4   use an *in vitro* model of erythromelalgia consisting of dorsal root ganglion neurons derived from

5   human induced pluripotent stem cells obtained from a patient (carrying the mutation F1449V) and a

6   control subject. We combine neurophysiology and computational modelling to focus on the Nav1.7

7   voltage gated sodium channel, which acts as an amplifier of the receptor potential in nociceptive

8   neurons and plays a critical role in erythromelalgia due to gain of function mutations causing the

9   channel to open with smaller depolarisations.

10   Using extracellular recordings, we found that the scorpion toxin OD1 (a Nav1.7 channel opener)

11   increases dorsal root ganglion cell excitability in cultures obtained from the control donor, evidenced

12   by an increase in spontaneous discharges, firing rate and spike amplitude. In addition, we confirmed

13   previous reports of voltage clamp experiments concerning an increase in spontaneous discharge in

14   the patient cell cultures and the analgesic effects of the Nav1.7 blocker PF-05089771. Our findings

15   are explained with a conductance-based model of the dorsal root ganglion neuron, exploring its

16   behaviour for different values of half activation voltage and inactivation removal rate of the Nav1.7

17   current. Erythromelalgia was simulated through a decrease of the Nav1.7 half activation voltage,

18   turning previously subthreshold stimuli to pain-inducing, and successfully counteracted with the

19   channel blocker. The painful effects of OD1 were simulated through a quicker removal of Nav1.7

20   inactivation that reproduced the effects of the toxin not only on the spike frequency but also on its

21   amplitude.

22   **New & Noteworthy**

23   We investigate Nav1.7 channel gating mechanisms in human iPSC-derived dorsal root ganglion cell

24   cultures using multielectrode array recordings. The scorpion toxin OD1 increases firing frequency

25   and spike amplitudes whilst the analgesic PF-05089771 decreases or even abolishes spontaneous

26   activity. The antagonistic effect of these compounds is explained with a computational model to

27   reach deeper understanding of changes in channel kinetics in erythromelalgia, a chronic pain

28   disorder.

29   **Introduction**

30   Chronic pain is a global healthcare problem, particularly affecting elderly people, women and

31   persons with lower socio-economic status (Van Hecke et al., 2013). It is one of the most common

32   reasons for physician consultation in developed countries, interfering with quality of life and causing

33   large socio-economic impacts that include significant loss of working hours and the need of clinical

34   care. Current therapies have limitations in their effectiveness and side effects, creating an urgent

35   need to develop more precise and effective treatments for pain management (Khouzam, 2000). In

36   this study we focus on the voltage dependent properties of the sodium channel Nav1.7 that is

37   implicated in inherited erythromelalgia, a vascular peripheral pathology causing attacks of severe

38   chronic pain (McDonnell et al., 2016).

39   Pain evoked spiking activity starts in peripheral terminals of dorsal root ganglion (DRG) neurons. The

40   central extensions of these neurons form the Aδ and C fibres which establish glutamatergic synapses

41   onto second order neurons within the spinal cord. They can be stimulated by mechanical, thermal or

42   chemical stimuli as well as by inflammatory mediators. Distributed throughout the body (skin,

43   viscera, muscles, joints, meninges), they carry noxious sensory information into the central nervous

44   system (Serpell, 2006). The recent discovery of nociceptive Schwann cells has changed the notion of

45   bare nerve terminals being the starting point of pain sensation, and introduced the concept of a glio-

46   neural end organ in the skin that transmits nociceptive information to the nerve, resembling the

47   specialized receptor cells found in other sensory systems (Abdo et al., 2019).

48   DRG neurons express several types of voltage gated sodium channels with different properties (Lera

49   Ruiz and Kraus, 2015). The opening of Nav1.7 channels requires smaller depolarisations than other

50   types of sodium channels, being more easily activated by the graded generator potentials. For this

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51   reason it is considered to be a threshold channel that acts as an amplifier of the receptor potential,

52   increasing the probability of triggering a spike via the activation of other, higher threshold sodium

53   channels such as Nav1.8. This amplification property makes Nav1.7 a major contributor to pain

54   signalling in humans (Dib-Hajj et al., 2013).

55   Erythromelalgia is caused by gain‐of‐function mutations of the gene SCN9A, which encodes for the

56   Nav1.7 channels pain (McDonnell et al., 2016). Different types of mutations have in common the

57   need for less depolarization to open the channel than in the wild type, resulting in a decrease of the

58   rheobase (i.e., minimal amplitude of an infinite depolarizing current to evoke a spike) (Cao et al.,

59   2016). DRG neurons fire spontaneously in erythromelelgia patients, although normally these cells

60   are silent unless receiving strong peripheral pain stimulus.

61   In this study, we have used extracellular recordings of spontaneous activity in DRG neuron cultures

62   derived from human induced pluripotent stem cells (hiPSCs) obtained from an erythromelalgia

63   patient and a control subject to study the voltage dependent gating properties of Nav1.7 channels.

64   To start, the effects on the neuronal firing of two different pharmacological compounds -a pain

65   eliciting scorpion toxin (OD1, Motin et al., 2016) and an analgesic drug (PF-05089771, Cao et al.,

66   2016)- were assessed in these cultures using multi electrode array (MEA) recordings. We then

67   present a simple conductance-based computational model of the DRG neuron to explain our findings

68   in terms of the Nav1.7 gating process, focusing on the channel activation (opening) and repriming

69   (i.e., recovery from inactivation).

70   **Materials and Methods**

71   **Cells and MEA recordings**

72   For this study we used hiPSCs from a control subject (cell line AD3) and an erythromelalgia patient

73   (cell line RCi002-A, carrying the mutation F1449V) made available at the European Bank for induced

74   pluripotent stem cells (EBiSC). The cells were differentiated into sensory neurons using a small

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75   molecule based protocol described previously (Cao et al., 2016, and references therein). Once they

76   reached the stage of DRG neurons, the cells were re-plated in 24 wells MEA plates (MEA700,

77   Multichannel Systems, Reutlingen, Germany) where the spontaneous activity was recorded after 10

78   weeks of maturation. Each well contains 12 circular electrodes (100 μm diameter, 700 μm electrode

79   pitch), making a total of 288 electrodes per plate. The large distance between electrodes makes it

80   very unlikely that the activity originating from an individual neuron will be detected by adjacent

81   electrodes. On the other hand, due to the relatively large cell density in these cultures, which

82   organize in structures called neural rosettes (e.g., Wilson and Stice, 2006), it is quite likely that one

83   electrode can detect the activity of more than one neuron (up to four in our data sets, although

84   most channels had only one or two).

85   We compared the spontaneous activity of DRG neurons obtained from a control subject (plated in

86   18 wells, making a total of 216 electrodes in MEA 1) with an erythromelalgia patient (plated in 24

87   wells, making a total of 288 electrodes in MEA 2). In addition we used a third MEA plated with

88   diseased cells stimulated with higher concentration of potassium (from 4.16 mM in the growing

89   medium to 6 mM) to enhance spontaneous activity (plated in 24 wells, making a total of 288

90   electrodes in MEA 3). This allowed us to find more active neurons following the addition of PF-

91   05089771 to make paired comparisons before and after this treatment that tends to suppress the

92   firing in many channels. Each well constitutes an independent cell culture.

93   The cultures were treated with OD1 (100 nM) in the control subject (MEA 1) and with PF-05089771

94   (100 nM) in the patient (MEAs 2 and 3). The activity was recorded for 5 minutes immediately before

95   the application of each substance and compared with a recording of the same duration performed 5

96   minutes after the onset of drug exposure. Wells contained 200 μl of growing medium and 5 μl drops

97   were added to apply the treatments. The experimental doses were selected to be in a near

98   saturation range to ensure a strong effect, based on dose response curves published previously (Cao

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99   et al., 2016 for PF-05089771; Motin et al., 2016 for OD1). Both compounds were purchased from

100   Tocris (Bio-Techne, Abingdon, UK).

Spike sorting and pairing of units

102   Recordings were performed with the Multiwell-MEA-System and the software Multi Channel

103   Experimenter (Multi Channel Systems, Reutlingen, Germany). Each channel was band-pass filtered

104   (100 to 3500 Hz) and acquired with a sampling rate of 20 kHz.

105   The raw voltage traces were first plotted and inspected using a zoom tool to discard artefacts and

106   confirm the existence of spikes. The voltage time series were then fed into the Matlab toolbox

107   Waveclus (Quian Quiroga et al., 2004) to perform spike sorting in all active channels. In the toolbox,

108   the continuous data were filtered again with a non-causal band pass filter between 300 and 3000 Hz

109   and the firing times were detected with an amplitude threshold. We used a dual threshold (i.e.,

110   picking deflections in both up and down directions) set to 5 median absolute deviations of the

111   filtered voltage signal with a refractory period of 2 ms to avoid double annotations due to fast

112   voltage oscillations in the vicinity of the threshold. Spikes were aligned to their maximum, after

113   interpolating the waveforms to locate the peak time more accurately. The toolbox uses a wavelet

114   based method for feature extraction, and the grouping of spikes into clusters is done with super-

115   paramagnetic clustering (Blatt et al., 1996), a stochastic algorithm that does not assume any

116   particular distribution of the data. In few cases the dual threshold created mistakes in the detection,

117   so we decided to use a single threshold, kept for the units that corresponded to the same neuron

118   before and after a given treatment. We consider this spike sorting strategy as supervised, always

119   following the criterion of the biologist as ground true. The firing times and voltage cut outs of all

120   units were stored to document the parameters and quality of the spike sorting in each channel.

121   Units that were active both before and after each pharmacological treatment were identified. The

122   only set-in-stone criterion for deciding if they are the same cell is the coordinates of the current

123   sources, using high density MEAs (Hilgen et al., 2017). As our data were recorded with low density

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124 MEAs, we cannot provide this level of certainty, but assumed that they corresponded to the same

125 neuron if the wave shape remained similar across recordings performed within few minutes of each

126 other. Possible ambiguities were minimized by the fact that most channels yielded only one or two

127 active neurons. These pairs were used to investigate the changes in spike frequency and amplitude

128 caused by the pharmacological treatments (Figs 1 and 2). If we were reasonably convinced that a

129 given unit corresponded to the same neuron before and after treatment, we designated the two

130 recordings as a pair. In the case of the control cultures, few neurons had spontaneous discharges

131 and most cells started to fire only after the OD1 treatment. Conversely, in the case of the patient

132 cultures, several neurons were active before, but few remained active after the application of PF-

133 05089771. In that case, we made an additional recording after increasing the spontaneous activity

134 with potassium, as mentioned above. This manipulation allowed to get more active neurons in

135 control conditions, and then more neurons remained active after the treatment to configure pairs,

136 although we did not pool the data recorded with and without higher potassium concentration.

137 For statistical comparisons between two conditions we used the Wilcoxon signed rank test (for

138 paired values) or the Mann–Whitney U test (for unpaired values). We also used cross correlation

139 histograms of the spike times recorded from the same channel to assess whether neurons tend to

140 fire at fixed delay from another.

141 **Numerical simulations**

142 We performed numerical simulations of the membrane potential and sodium channels gate

143 parameters of a single DRG neuron using the software NEURON (www.neuron.yale.edu), e.g., Choi

144 et al., 2011). The integration method was the IDA algorithm (e.g., Carnevale, 2007) with a fixed time

145 step of 0.025 ms (i.e., 40 time steps per ms). Our purpose is to explain the changes in frequency and

146 amplitude induced by the drug treatments covered in the previous section. In this context we

147 adopted a parsimonious approach to keep the model as simple as possible, using Hodgkin and

148 Huxley (HH) type of Na^+ and K^+ currents (Ermentrout and Terman, 2010) with parameters (Table 1)

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149   adapted to be plausible for mammal cells (Krouchev et al., 2015) plus a single Nav1.7 type current

150   added using the Channel Builder GUI (McDougal et al., 2017).

151   The two different types of sodium channels that we included differed only in their parameters (Table

152   1), the form of the equations was the same (S1 Appendix). The first, with larger conductance (0.3

153   S/cm^2) and a more depolarized value of half activation voltage (d = -40 mV) resembles a HH type

154   high threshold Na$^+$ channel (like Nav1.8 in mammals). The second type of Na$^+$ channel, with a half

155   activation voltage shifted in the hyperpolarizing direction (d = -55 to -60 mV) and a smaller value of

156   maximum conductance (0.1 S/cm^2), represented a Nav1.7. It had faster opening/closing and

157   activating/inactivating rates and a more depolarized value of half inactivation voltage removal than

158   the HH type channel (Table 1). These changes allowed to create a rapidly activating but slowly

159   repriming (slow recovery from inactivation) current, resembling Nav1.7. To simulate erythromelalgia

160   we varied the half activation voltage of Nav1.7 from -55 to -60 mV. To simulate PF-05089771 effect,

161   we varied the maximum conductance of Nav1.7 from 0.1 to 0.07 S/cm^2 (Figs 3 and 4) and to simulate

162   OD1 effects (Figs 5, 6 and 7) we increased 10 times the parameter A_h that multiplies the inactivation

163   removal rate a_h. Model parameters are provided in Table 1, the equations that are most relevant for

164   our results appear in section Results and all model equations are listed in S1 Appendix.

165   Step depolarisations of 0.04 nA lasting 60 ms were used to evaluate the effect of the half activation

166   voltage (d_h) and the inactivation removal rate (a_h) on the firing frequency of the spike (Figs 3 and 5).

167   To estimate more reliably the spike frequency and amplitude by averaging over many spikes we used

168   depolarization steps lasting 5 s (Figs 4 and 6).

169   In order to assess for bifurcations in the dynamical system, we performed simulations of the

170   maximum and minimum values of the voltage oscillation as a function of the amount of external

171   current injected (i.e., a one dimensional bifurcation diagram, e.g., Fig 3 in Doi et al., 2001, for d_m = -

172   58 mV and g_{max} = 0.1 S/cm^2.

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173   All figures of the article were created with Matlab (R2018b, The MathWorks, Inc., Natick,

174   Massachusetts, United States) and finalized with Adobe illustrator (Adobe Inc.).

Results

Experimental recordings

177   We compared the spontaneous activity of DRG neurons obtained from a control subject (cell line

178   AD3, n = 18 cultures) with an erythromelalgia patient (cell line RCi002-A, n = 24 cultures). The

179   percentage of electrodes showing activity was 1.85 % in the control and 5.9 % in the disease

180   cultures. After spike sorting we found 30 spontaneously active units in the patient and only 7 in the

181   control. The firing frequency and amplitude of these units were not significantly larger in the disease

182   cultures, implying that although much more units are active, they do not necessarily fire faster or

183   have spikes of larger amplitude. It is worth mentioning that the fastest neurons and the tallest spikes

184   were found in the disease cultures, but the distributions were not significantly different because

185   slow units with small amplitude were present in both groups. The larger number of spontaneously

186   active neurons found in the disease cultures is consistent with the results of a previous study (Cao et

187   al., 2016).

188   The cultures from the control subject were treated with OD1 (100 nM), resulting in the percentage

189   of active channels increasing from 1.85 to 6.02 % and a concomitant increase in the number of active

190   units, from 7 to 24. An example of the voltage data before and after treatment is presented in Fig 1-

191   A, note the large increase in spontaneous activity. The same picture was observed in an

192   erythromelalgia culture conducted just as a verification (not shown).

193   Pairing all the units that were active before and after OD1 application in the control cells (n = 7), we

194   found that both the spike frequency and amplitude increased significantly (Fig 1-B). Although the

195   increase in amplitude was not dramatic (20 % in average), it was consistently found in every pair of

196   units. The extent of the increase in firing frequency was more pronounced (70 %). The unpaired units

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197 that were initially silent and started to fire after the OD1 were not significantly different in rate and

198 amplitude from those already active before the treatment.

199 The cells from the patient were treated with PF05089771 (100 nM). The percentage of channels with

200 spiking activity fell from 5.9 to 2.08 %. An example of the raw voltage trace before and after

201 treatment is presented in Fig 2-A, note that the spiking activity is decreased, although this particular

202 recording was done in the presence of higher K^+ concentration.

203 Pairing the units that were active before and after the treatment (n=6), we found that the spike

204 frequency decreased significantly but the amplitude did not change (Fig 2-B). This result was

205 confirmed with a larger number of paired units (n=16) in a MEA plate previously exposed to high

206 potassium concentration (Fig 2-C). In both cases the unpaired units that stopped firing as a result of

207 the PF05089771 application were not significantly different in rate and amplitude to the ones that

208 remained active after treatment.

209 In order to assess for possible functional connectivity, we calculated cross correlation histograms

210 between the firing times of the neurons that were recorded from the same electrode. No histogram

211 peaks at fixed latency were found, which is compatible with the established notion that DRG

212 neurons do not form synaptic contacts between them.

213 **Numerical simulations**

214 In this subsection we present the results obtained with the computational model of the single DRG

215 neuron explained in section Methods, including the equations directly related with the quantities

216 plotted in the figures. The equations of all currents are provided in S1 Appendix.

217 Our DRG neuron was simulated as a cylindrical soma of 30 μm diameter and height. The ionic

218 conductances are based on a HH model with mammalian-plausible parameters plus an additional

219 Na^+ current with the features of a Nav1.7 (Table 1). The disease and health conditions were

220 simulated through changes in the Nav1.7 half-activation voltage parameter (d_m in equation 1). In

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221   erythromelalgia patients, this parameter is shifted up to 15 mV in hyperpolarizing direction (Cao et

222   al., 2016), causing a decrease in the level of depolarization needed to activate the Nav1.7 current.

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General parameter set	
L (μm)	30
d (μm)	30
cm (μF/cm^2)	1
gNa_hh (S/cm^2)	0.3
gNav1.7_hh (S/cm^2)	0.1
gK_hh (S/cm^2)	0.15
gL_hh (S/cm^2)	3.00E-05
eL_hh (mV)	-65
eK (mV)	-90
eNa (mV)	60
Initial V (mV)	-75
dt (ms)	0.025
Temp (Celsius)	37

K HH parameters	
Opening (a$_m$)	
A (ms^{-1})	0.1
k (ms^{-1})	0.1
d (mV)	-55
Closing (b$_m$)	
A (ms^{-1})	0.125
k (ms^{-1})	-0.0125
d (mV)	-65

Na HH parameters	
Opening (a$_m$)	
A (ms^{-1})	1
k (ms^{-1})	0.1
d (mV)	-40
Closing (b$_m$)	
A (ms^{-1})	4
k(ms^{-1})	-0.055
d (mV)	-65
Inactivation removal (a$_h$)	
A (ms^{-1})	0.07
k (ms^{-1})	-0.05
d (mV)	-65
Inactivation establishment (b$_h$)	
A (ms^{-1})	1
k (ms^{-1})	-0.1
d (mV_	-35

Nav1.7 parameters	
Opening (a$_m$)	
A (ms^{-1})	13.78
k (ms^{-1})	0.1
d (mV)	-58
Closing (b$_m$)	
A (ms^{-1})	55.11
k (ms^{-1})	-0.055
d (mV)	-65
Inactivation removal (a$_h$)	
A (ms^{-1})	0.92
k (ms^{-1})	-0.05
d (mV)	-40
Inactivation establishment (b$_h$)	
A (ms^{-1})	8.76
k (ms^{-1})	-0.1
d (mV)	-35

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226   **Table 1. Default parameter values used for the simulations.** Particular cases are indicated in the

227   corresponding figures.

228   The rate of Nav1.7 channel opening a_m is given by

229    $a_m = \frac{A_m\,(k_m\,(v-d_m))}{1-e^{-k_m\,(v-d_m)}}$ (1)

230   where v is the membrane potential, d_m is the half-activation voltage, and k_m and A_m are constants

231   (Table 1). The opening and closing dynamics of the current is given by the differential equation

232    $\frac{dm}{dt} = a_m(1-m) - b_m m$ (2)

233   where m is the opened state of the channel, $1-m$ the closed state and b_m the closing rate.

234   The conductance is a cubic function of m,

235    $g_{Nav1.7} = g_{max}\,m^3 h$ (3)

236   where h is the inactivation process and g_{max} the maximum conductance.

237   In Fig 3 we show the effects of varying d_m (Equation 1) in the model neuron by displaying the

238   response to a depolarizing current step of 0.04 nA lasting 60 ms. This level of current is slightly above

239   the firing threshold at d_m = -58 mV and g_{max}= 0.1 S/cm^2, which is 0.037 nA. At this threshold the one

240   dimensional bifurcation diagram (not shown) presents a discontinuity that corresponds to a Hopf

241   bifurcation (see section Discussion). The upper blue trace represents a normal subject (d_m = -55 mV)

242   where the stimulus does no elicit pain (i.e., no spiking). The pink traces of the middle row represent

243   mild erythromelalgia (d_m = -58 mV) where the same stimulus is painful (spiking in the left panel) and

244   can be successfully treated by blocking 20% of the Nav1.7 channels, i.e., decreasing the maximum

245   conductance (g_{max} in Equation 3) from 0.1 to 0.08 S/cm^2 (no spiking in the right panel). In the red

246   traces of the lower row we simulate severe erythromelalgia (d_m = -60 mV), showing faster spiking

247   rate (left) and spontaneous spikes occurring before and after the stimulus. In this case, blocking 20%

248   of the Nav1.7 channels decreases the firing but is not able to stop it (middle panel). The pain eliciting

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249 firing can be stopped by blocking a larger percentage of Nav1.7 channels (30% with g_{max} = 0.07

250 S/cm^2, right panel).

251 In Fig 4 we provide a qualitative frame to understand the situations described in the previous figure.

252 Using a heat map, the spike rate of the neuron model (stimulated with 0.04 nA depolarizing current)

253 is represented with a color bar for different values of maximum conductance g_{max} (Equation 3,

254 abscissas) and half activation voltage parameter d_m (Equation 1, ordinates) of the Nav1.7 current.

255 The white circles with numbers on the heat map indicate the coordinates (g_{max} and d_m) used for the

256 simulations depicted in Fig 3. The numbers inside the circles of Fig 4 correspond to the numbers in

257 the panels of Fig 3. Erythromelalgia mutations cause pain through a downward shift toward the red

258 values of the heat map (e.g., trajectories 1 to 2 and 2 to 4). Conversely, the treatment with Nav1.7

259 blockers (e.g., PF05089771) alleviates pain through a shift to the right, towards the blue values (e.g.,

260 trajectories 2 to 3 and 4 to 5 and 6).

261 In a second series of simulations we explored the inactivation properties of Nav1.7 channels

262 (parameter sets displayed in Table 1). The scorpion toxin OD1 has been reported to increase the rate

263 at which Nav1.7 inactivation is removed after being established (Motin et al., 2016). In order to

264 emulate this effect in the model, we increased 10 times the removal rate of Nav1.7 inactivation (a_h

265 in Equation 4).

266 The inactivation removal rate is given by

267 $a_h = A_h\, e^{k_h(v - d_h)}$ (4)

268 where v is the membrane potential, k_h and A_h are constants (Table 1) and d_h is the half-inactivation

269 removal parameter. The inactivation process dynamic is given by the temporal derivative of h

270 $\frac{dh}{dt} = a_h(1 - h) - b_h h$ (5)

271 where $1-h$ is the inactivated state and h is the non-inactivated state that allows ionic conduction.

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272   To increase a_h we increased the multiplicative constant A_h by 10 times (Equation 4). This change

273   caused the model neuron to fire in response to a depolarizing pulse of 0.04 nA that was not effective

274   before (upper panels in Fig 5) and elicited a faster firing rate if the neuron was already active in the

275   control condition (lower panels in Fig 5).

276   The faster removal of Nav1.7 inactivation (A_h varied from 0.92 to 9.2) also increased the model spike

277   amplitude to a moderate amount in the simulation after OD1 (red plot in Fig 6), as observed in the

278   extracellular recordings (Fig 1-B). In Fig 7-A, we show plots of m (thin full lines) and h (dashed lines)

279   during a spike, in a simulation before (blue plots of left panel) and after OD1 (red plots of right

280   panel). Both panels display the Nav1.7 current with full thick lines. Note that in the simulation after

281   OD1, Nav1.7 has larger amplitude, and the values of h are higher, with a faster return to 1 after the

282   negative peak. These large changes of h were not observed in the HH type high threshold sodium

283   current, although the current amplitude was slightly increased (Fig 7-B).

284   **Discussion**

285   In this study, we performed to our knowledge the first test of the scorpion toxin OD1 in hiPSC-

286   derived DRG neurons. This toxin enhances the recovery from fast inactivation of the threshold

287   current Nav1.7 (Motin et al., 2016). Our extracellular recordings also confirmed previous findings of

288   Cao et al. (2016) with patch clamp regarding the increased spontaneous firing of erythromelalgia

289   DRG cells and the analgesic effects of PF-05089771. This drug was shown to be clinically effective to

290   control heat induced pain attacks by stabilizing the voltage sensor domain of the Nav1.7 channel in a

291   non-conducting conformation. The effects of both compounds were explained using a conductance-

292   based computational model, comparable with Choi et al. (2011) but simpler and particularly

293   focalized on the issue of pain signalling. In this section we first discuss our main findings and

294   emphasize the usefulness of extracellular recordings to observe changes in ionic currents. To close

295   the discussion, we provide a short remark about the importance of the interaction between

296   different sodium currents in erythromelalgia.

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297   **Effects of erythromelalgia, PF-05089771 and OD1**

298   Cao et al. (2016) performed voltage clamp experiments in whole-cell configuration in DRG cells

299   derived from erythromelalgia patients and control subjects. Despite the heterogeneity of the

300   samples, they found a significantly higher proportion of spontaneously firing cells in patients

301   compared to those from control donors. Moreover, the patient's cells showed a lower rheobase and

302   reached higher firing rates in response to stimulation with current steps of increasing amplitudes.

303   These findings point to the existence of elevated excitability in erythromelalgia cells, a fact that we

304   confirmed in the present study by showing higher prevalence of spontaneous activity in extracellular

305   MEA recordings (section Results).

306   Spontaneous firing in our cultures is due only to intrinsic cell excitability, as there are no reports

307   about the formation of synapses between DRG cells *in vitro*. Accordingly, we did not find evidence of

308   functional connections in cross correlation histograms between neuronal firing times. Furthermore,

309   in dissociated cell cultures the development of a synaptic network is usually accompanied by the

310   emergence of population bursts (Maeda et al., 1995) which we did not observe here. DRG glia cells

311   are not present in the cultures, so their reported contribution to abnormal neuronal activity in *in*

312   *vitro* studies done on intact DRG (Belzer and Hanani, 2019) can also be ruled out.

313   Regarding the effects of Nav1.7 channel blockers, Cao et al. (2016) reported a dose dependent

314   reduction in spontaneous firing and an increase in the action potential rheobase. We were able to

315   reproduce the first finding on spontaneous firing with PF-05089771 (Fig 2), supporting the fact that

316   the excitability of the cells plummets after the treatment, in accordance with the reported clinical

317   efficacy of the drug (Cao et al., 2016). We did not, however, observe significant changes in spike

318   amplitudes before and after drug treatment.

319   The effect of OD1 (and other synthetic toxin analogs) on the gating properties of the Nav1.7 sodium

320   channels was studied by Motin et al. (2016) using voltage clamp whole cell and single channel

321   recordings in Chinese hamster ovary (CHO) cells expressing human Nav1.7 channels. They found that

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322   the decay phase of the Nav1.7 channel opening slows down in a concentration-dependent manner

323   in the presence of these toxins. Using a paired-pulse protocol in the voltage clamp configuration, the

324   authors demonstrated an enhancement of Nav1.7 recovery from fast inactivation. Single channel

325   recordings showed that the mean open time of the channels was not substantially changed by the

326   toxin, but the channels exhibited a prolonged flickering behavior between open and closed states,

327   which enabled a more efficient inactivation removal. These experiments pointed to a voltage-sensor

328   trapping interpretation, in which the toxin prevents a conformational change in the domain IV

329   voltage sensor, as the main cause for the observed prolongation in the current duration. Motin et al.

330   (2016) also found that the current-voltage relationships of Nav1.7 are shifted to more negative

331   potentials in the presence of OD1, but this effect has a small magnitude (-3 mV), in accordance with

332   earlier observations in *Xenopus laevis* oocytes with Nav1.7 channels expression (Maertens et al.,

333   2006).

334   Our findings corroborate those of Motin et al. (2016). Indeed, we observed an increase in the

335   proportion of electrodes showing spontaneous firing in the presence of OD1, as well as an increase

336   in firing rate in cells that did fire before adding the toxin. In addition to the firing frequency rise, we

337   found a consistent increase in the spike amplitude after OD1, in contrast with the PF-05089771

338   treatment which affected only the firing rate. These larger extracellular spikes are in accordance

339   with the increase in peak current observed in the presence of OD1 in voltage clamp experiments

340   (Maertens et al., 2006; Motin et al., 2016). Since the amplitude of the current generated by the

341   opening of a single channel was not modified by OD1 (Motin et al., 2016), the size increase of the

342   extracellular spikes must be due to the dynamics of the gating process. In the next section, we

343   address the numerical simulations to offer an explanation for the changes in spike rate and

344   amplitude described above.

345   **Numerical simulations**

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346   The computational model that we present here is able to provide satisfactory explanations at a

347   qualitative level for the main experimental findings of our study and for previous studies on

348   erythromelalgia and the effects of pharmacological manipulations on Nav1.7 channels. We did not

349   attempt to perform a complete exploration of the parameter space but all values are biologically

350   plausible. In the absence of stimulation the membrane potential tends to its fixed point which is the

351   resting potential. By injecting an amount of current also plausible for a patch clamp experiment

352   (0.037 nA for d_m = -58 mV and g_{max} = 0.1 S/cm^2), the system reaches a Hopf bifurcation (e.g.,

353   Ermentrout and Terman, 2010), the critical point corresponding to the spike threshold. At a critical

354   point, the stability of the system switches and a periodic solution of repetitive spiking arises, eliciting

355   pain. In the dark blue area of no firing of Fig 4 the membrane potential is at a fixed point (resting

356   potential). The periodic solution occurs in the areas having other colors (including lighter tones of

357   blue). The Hopf bifurcation happens at the limit between the dark blue and the other colors, roughly

358   resembling a diagonal trajectory in the heat map. The descending transition from 1 to 2 and 4

359   exemplifies the trajectory towards pain in erythromelalgia and the horizontal transitions from 2 to 3

360   and from 4 to 5 and 6 depict the analgesic path triggered by PF-05089771.

361   While the decrease of Nav1.7 maximum conductance accounted for the analgesic effects of PF-

362   05089771, OD1 effects on the firing rate were reproduced by enhancing the constant governing the

363   removal rate of the inactivation a_h (Fig 5). The interpretation of these results is quite straight

364   forward, having fewer available Nav1.7 receptors reduces the firing by interfering with the

365   amplification of subthreshold potentials performed by the channel, while the faster inactivation

366   removal increases the current size and duration (Fig 7), leading to increased spiking and excitability.

367   This is in line with the proved analgesic effects of Nav1.7 blockers (Cao et al., 2016) and with the

368   intense pain known to be experienced after a scorpion sting (e.g., Garfunkel et al., 2007).

369   In the case of spike amplitude, the explanation of the results requires a deeper elaboration. Starting

370   from the fact that spike depolarization is sustained by sodium currents, we can see that the

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371   maximum amplitude that a spike can potentially reach in the absence of inactivation is given by the

372   sodium equilibrium potential. The inactivation process will also limit the maximum value reached by

373   the current and the peak intracellular voltage, because the current starts to inactivate before the

374   opening process is completed. If this reasoning is correct, an increase of the inactivation removal

375   rate of Nav1.7 will create a larger spike, because more channels will be free from inactivation during

376   the critical period of the spike rising phase.

377   This last interpretation is exemplified in the numerical simulations of Figs 6 and 7. The consequences

378   of the faster inactivation removal rate of Nav1.7 after OD1 are noticeable in the higher values of the

379   process h, as well as in its faster return to 1 following the negative peak. The closer to 0 h reaches,

380   the more channels are inactivated, so a larger h value implies more open channels potentially

381   available, which is reflected in a larger Nav1.7 current (Fig 7-A). The high threshold Na^+ current also

382   grows slightly following enhancement of Nav1.7, without large changes of h. This increase is less

383   pronounced than for Nav1.7, but still contributes to increase the spike size because the total HH

384   type current is bigger. We must keep in mind that the extracellular spike is a filtered version of the

385   intracellular spike and we cannot necessarily compare the amplitude differences linearly. In addition,

386   Nav1.7 and Nav1.8 interact in a complex manner to regulate DRG neuronal excitability, as has been

387   shown in a previous modelling study of the rat HRV neurons (Choi and Waxman, 2011). Despite

388   these caveats, we can assume that in the situation depicted in our simulation both currents are

389   adding up to enhance the intracellular spike (Fig 6). This effect was captured in our extracellular

390   recordings (Fig 1).

391   **Model limitations**

392   We are interested in understanding the reason for the observed changes in spike rate and amplitude

393   created by the two compounds tested using a model simulation with biologically plausible

394   parameters. In this context, we validated our interpretation of biological facts with a numerical

395   simulation. In order to keep the model simple and with a clear biological meaning and physical unit

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396   for all variables, we opted for a modified HH scheme, which also has the virtue of being familiar to

397   neurobiologists. We included only one potassium current (non-inactivating, HH type), to avoid

398   unnecessary complexity. The only added current to the original HH scheme is the Nav1.7, because it

399   is strictly needed to implement the biological mechanisms under consideration. A detailed

400   quantitative modelling would require Ca^{2+} currents and several types of Na^+ and K^+ currents that

401   have been found in mammalian DRG cells (e.g., Rush et al., 2007; Scroggs and Fox, 1992; Choi and

402   Waxman, 2011; Newberry et al., 2016; Mandge and Manchanda, 2018; Zemel et al., 2018), and is

403   beyond the scope of this study. Although more refined kinetic models of the Nav1.7 sodium and

404   other channels have been proposed (e.g., Sigg, 2014), we think that we are adopting here a level of

405   simplification in the modelling process that is fitted for our purpose.

406   **Extracellular recordings and ionic currents**

407   Although extracellular recordings are typically used to detect firing times, they also have the

408   theoretical ability to report about what are considered to be intracellular features of the action

409   potential (Gold et al., 2006). We want to highlight this possibility of using extracellular recordings to

410   detect changes in ionic currents. If researchers first evaluate them together with voltage clamp data

411   for validation purposes, they can then benefit from the opportunity to observe more neurons

412   simultaneously. A small amplitude change of the DRG neuron spike might not play an important

413   physiological role in pain signalling, which is mainly conveyed by the spike rate, but we provided

414   here a proof of principle regarding the footprint of a current in the extracellular spike.

415   The fact that the extracellular spike did not show large changes in waveform shape with the

416   treatments (only scaling up moderately with OD1) facilitated our analysis, allowing reasonable

417   assumptions regarding neuron identities before and after the treatments, but this might not be the

418   case for other drugs or experimental systems (Hilgen, personal communication). It has been

419   reported that an intracellular spike broadening caused by K^+ current modulators can increase Ca^{++}

420   influx at the synaptic level in mouse hippocampal cultures (Vivekananda et al., 2017). In this context

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421   it makes sense to speculate about the possibility that, if a larger spike could elicit more

422   neurotransmitter release, then the spike amplitude would also contribute to pain signalling by

423   eliciting a discharge of higher frequency in the ascending spinal neurons.

424   **Importance of the interaction between sodium currents**

425   The interaction between sodium currents can have profound functional consequences, and the

426   clinical signs of erythromelalgia provide a striking example (Rush et al., 2006). As explained above, in

427   pain pathways Nav1.7 increases neuronal excitability mainly by amplifying the depolarization caused

428   by peripheral stimuli, allowing to trigger Nav1.8. This facilitates firing and pain, an effect that is

429   exaggerated in erythromelalgia patients. A key property for this firing to occur (as implemented in

430   our model) is that Nav1.8 does not inactivate until the voltage is quite high (e.g., around -35 mV). In

431   the sympathetic nervous system the situation is different because there are no Nav1.8 channels and

432   spikes are sustained by other sodium currents that inactivate at lower voltage (Rush et al., 2006).

433   Then, the excessive depolarization created by a mutated Nav1.7 in erythromelalgia can be sufficient

434   to trigger inactivation, terminating the firing. The excessively active mutated Nav1.7 increases firing

435   in HRV nociceptive neurons but blocks firing in the sympathetic nerves that mediate the contraction

436   of peripheral blood vessels. This results in excessive vasodilatation, creating the typical sign of red

437   extremities that originated part of the name to the disease (erythromelalgia could be translated as

438   "red neuralgia of the extremities"). The importance of the interaction between sodium currents is

439   highlighted by this example, because the same mutation produces opposite effects in the firing of

440   pain afferents and autonomic motor neurons.

441   **Figure captions**

442   **Fig 1. Effects of OD1 in nociceptive neurons.** (A) Voltage data of the most active channel in MEA 1

443   before (upper panel) and after (lower panel) OD1 (100 nM) application. Note the increase in rate

444   and amplitude of the spontaneous activity. (B) Rate and amplitude of paired neurons before and

445   after OD1 (100 nM) application. Corresponding pairs in both plots are color coded. Wilcoxon signed

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446   rank test p values are printed in each panel. The normalized changes of both indexes are highly

447   correlated (r = 0.78).

448   **Fig 2. Effects of PF05089771 in nociceptive neurons.** (A) Example of voltage data before and after

449   PF05089771 (100 nM). K^+ concentration was 6 mM. (B) Rate and amplitude of the neurons paired

450   before and after treatment. K^+ concentration was 4.16 mM. (C) Same as in B with a K^+ concentration

451   of 6 mM. Wilcoxon signed rank test p values are printed in panels B and C, corresponding pairs are

452   colour coded. The normalized changes in both indexes are poorly correlated (r = 0.21 in B and 0.53 in

453   C).

454   **Fig 3. Erythromelalgia and PF05089771 effects: simulation of membrane voltage.** Response of the

455   model neuron to a 60 ms depolarizing pulse of 0.04 nA for three different values of half activation

456   voltage d_m (in mV) and maximum conductance g_{max} (in S/cm^2). The numbers with circles in the panels

457   correspond to the numbers inside the white circles of Fig 4.

458   **Fig 4. Erythromelalgia and PF05089771 effects: heat map of the spiking activity.** The neuron model

459   spike rate is represented (color bar) for different values of maximum conductance g_{max} (abscissas)

460   and half activation voltage parameter d_m (ordinates) of the Nav1.7 current. The numbers in the

461   white circles correspond to the numbers in the panels of Fig 3. The dashed line on top indicates an

462   axis break.

463   **Fig 5. Simulation of OD1 effects on the spike rate.** Response to a 60 ms depolarizing pulse of 0.04

464   nA before (blue plots in left panels) and after (red plots in right panels) a 10 fold increase in the

465   inactivation removal rate a_h to simulate OD1 treatment. The value of d_m was varied from 57.8 mV

466   (upper panels) to 58 mV (lower panels), while A_h was varied from 0.92 (left panels) to 9.2 (right

467   panels).

468   **Fig 6. Simulation of OD1 effects on the spike amplitude.** Spikes with different removal rates of

469   Nav1.7 inactivation (parameter A_h = 0.92 in blue plot and 9.2 in red plot, with d_m = -58 mV). The red

470   spikes are slightly taller than the blue spikes (7 % average amplitude increase in a 5 s simulation).

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471   **Fig 7. Simulation of OD1 effects on Na$^+$ currents and their gating variables.** Values of m (thin full

472   lines), h (dashed lines) and Na$^+$ currents (thick full lines) during a spike. The currents were plotted in

473   10 x S/cm^2 and sign inverted for scale display purposes, as m and h always range from 0 to 1. A_h (in

474   Nav1.7 only) was set to 0.92 in the blue plots (simulation before OD1) and to 9.2 in the red plots

475   (simulation after OD1). (A) Nav1.7, note the higher h values after OD1. (B) High threshold Na$^+$

476   current, note that h is similar before and after OD1.

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547   **Supporting information**

548   **S1 Appendix. Equations Appendix.** Equations are from the HH model formulation provided by the

549   NEURON software and the Channel Builder GUI

550   (https://www.neuron.yale.edu/neuron/static/docs/chanlbild/main.html). Parameter values are

551   listed in Table 1 and provided with the corresponding figures.

552   **Acknowledgements**