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

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Short title:

Chronic pain and Nav1.7 gating in human nociceptive neurons

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Abstract

Chronic pain is a global healthcare problem with a huge societal impact. Its management remains generally unsatisfactory, with no single treatment clinically approved in most cases. In this study we use an *in vitro* model of erythromelalgia consisting of dorsal root ganglion neurons derived from human induced pluripotent stem cells obtained from a patient (carrying the mutation F1449V) and a control subject. We combine neurophysiology and computational modelling to focus on the Nav1.7 voltage gated sodium channel, which acts as an amplifier of the receptor potential in nociceptive neurons and plays a critical role in erythromelalgia due to gain of function mutations causing the channel to open with smaller depolarisations.

Using extracellular recordings, we found that the scorpion toxin OD1 (a Nav1.7 channel opener) increases dorsal root ganglion cell excitability in cultures obtained from the control donor, evidenced by an increase in spontaneous discharges, firing rate and spike amplitude. In addition, we confirmed previous reports of voltage clamp experiments concerning an increase in spontaneous discharge in the patient cell cultures and the analgesic effects of the Nav1.7 blocker PF-05089771. Our findings are explained with a conductance-based model of the dorsal root ganglion neuron, exploring its behaviour for different values of half activation voltage and inactivation removal rate of the Nav1.7 current. Erythromelalgia was simulated through a decrease of the Nav1.7 half activation voltage, turning previously subthreshold stimuli to pain-inducing, and successfully counteracted with the channel blocker. The painful effects of OD1 were simulated through a quicker removal of Nav1.7 inactivation that reproduced the effects of the toxin not only on the spike frequency but also on its amplitude.

New & Noteworthy

We investigate Nav1.7 channel gating mechanisms in human iPSC-derived dorsal root ganglion cell cultures using multielectrode array recordings. The scorpion toxin OD1 increases firing frequency and spike amplitudes whilst the analgesic PF-05089771 decreases or even abolishes spontaneous

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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).

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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.

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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).

101 **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.

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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.).

175 Results

176 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.

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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.

223

224

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

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

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

234 The conductance is a cubic function of m,

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

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).

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

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

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).

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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).

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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).

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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.

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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.

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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).

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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.

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552 ## Acknowledgements

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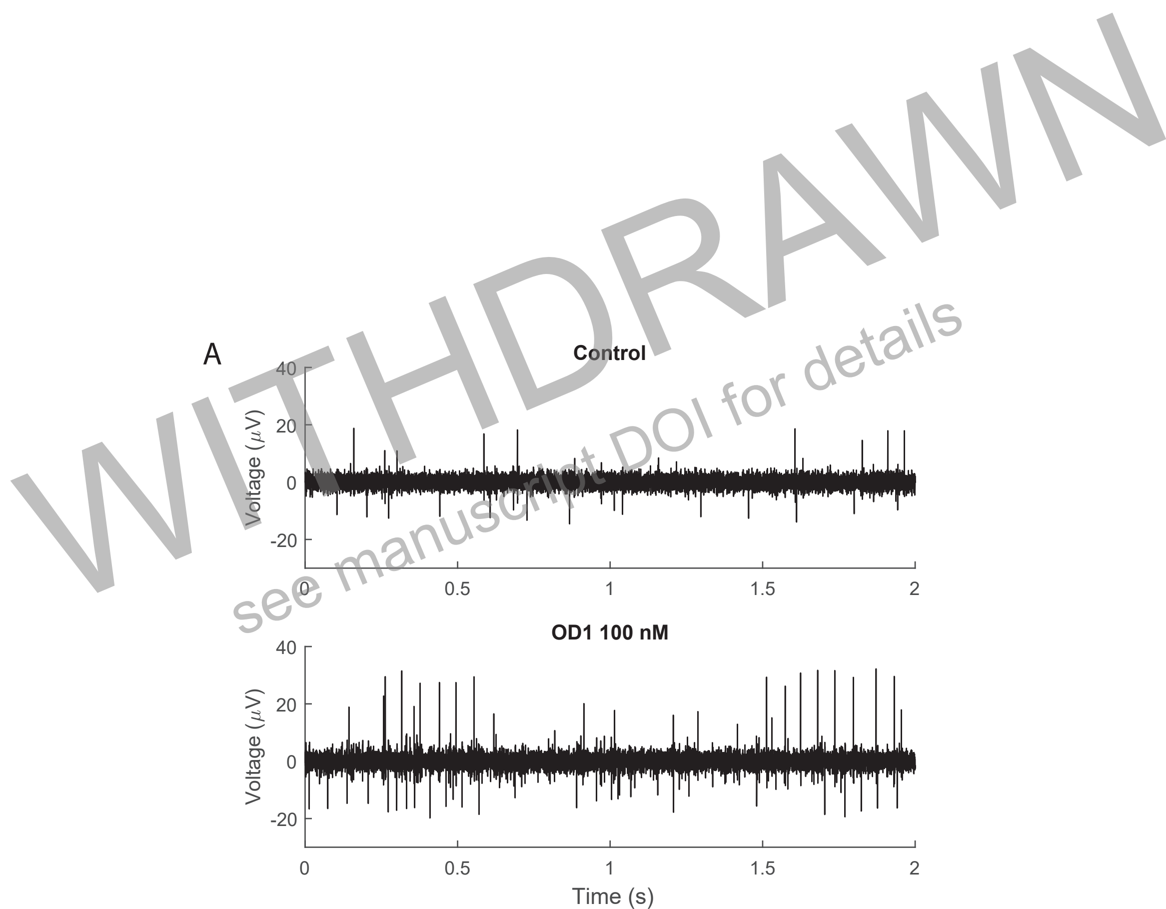

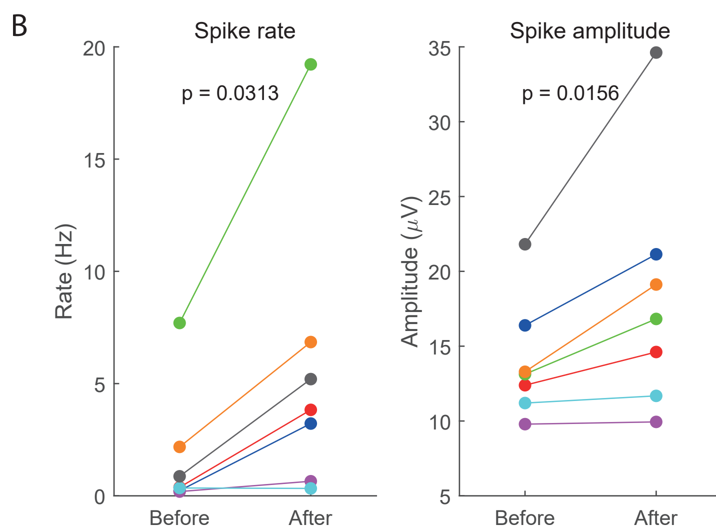

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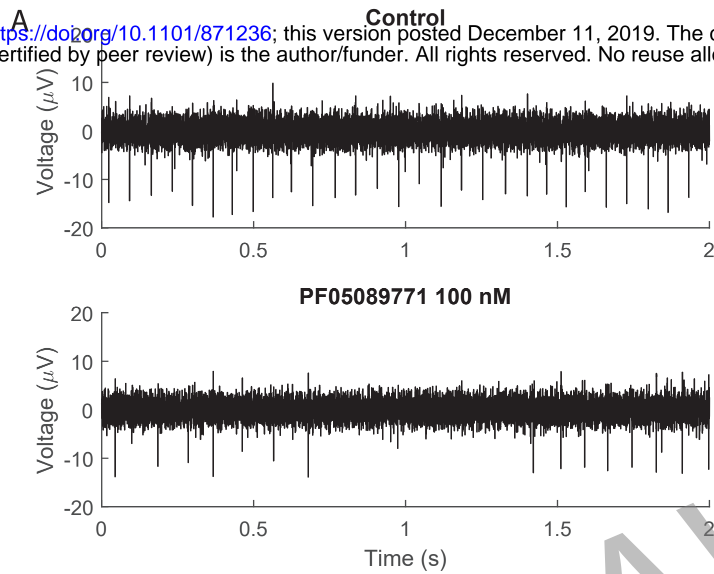

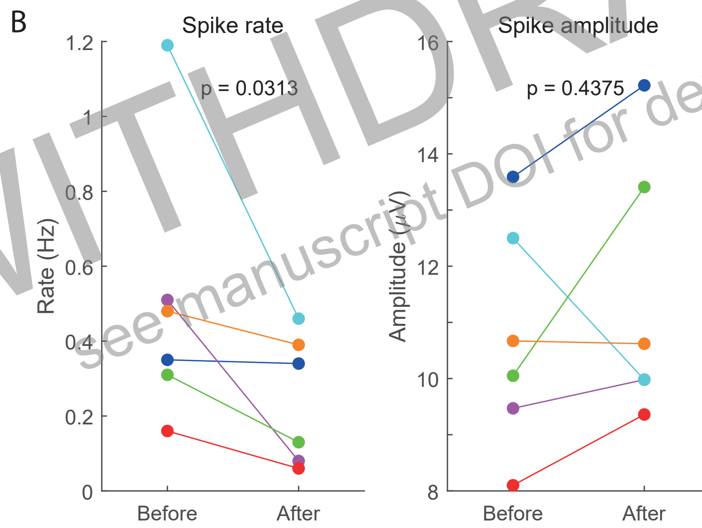

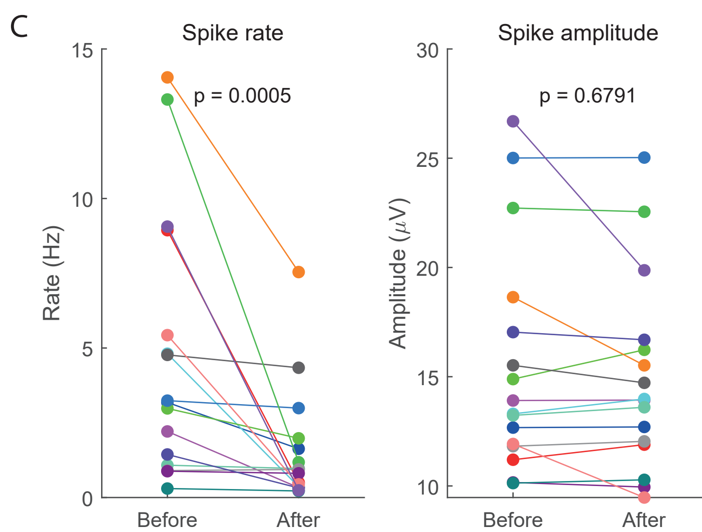

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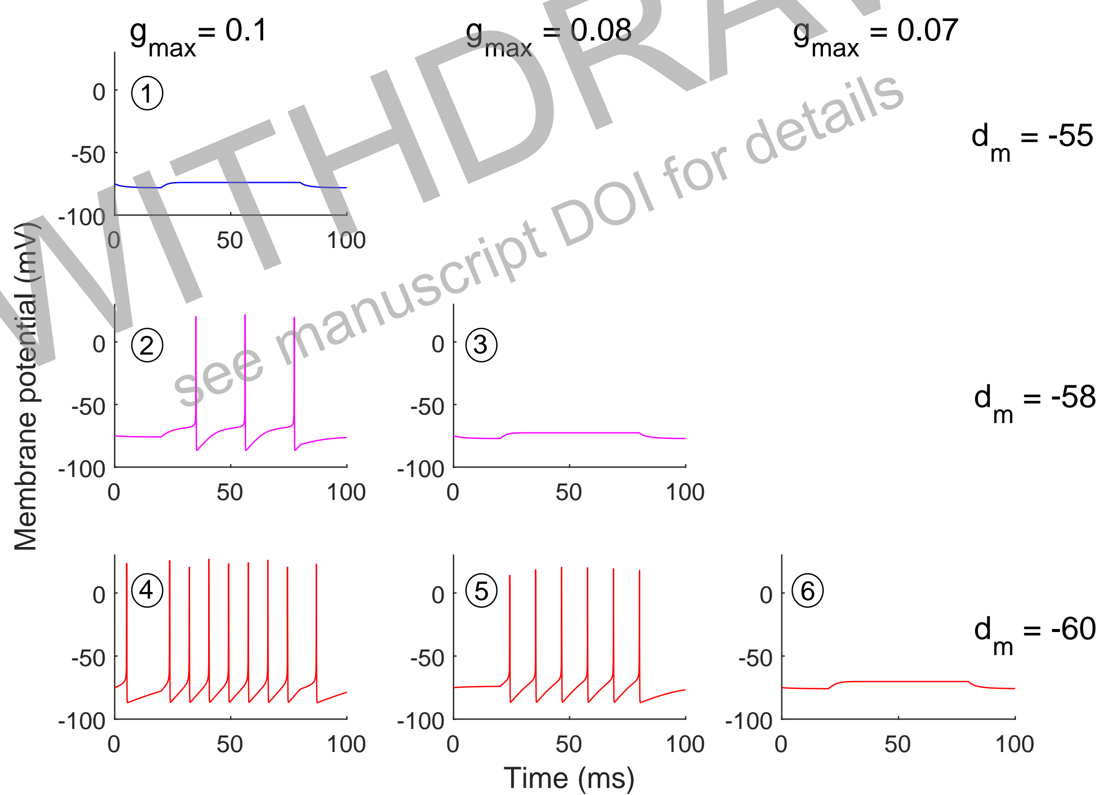

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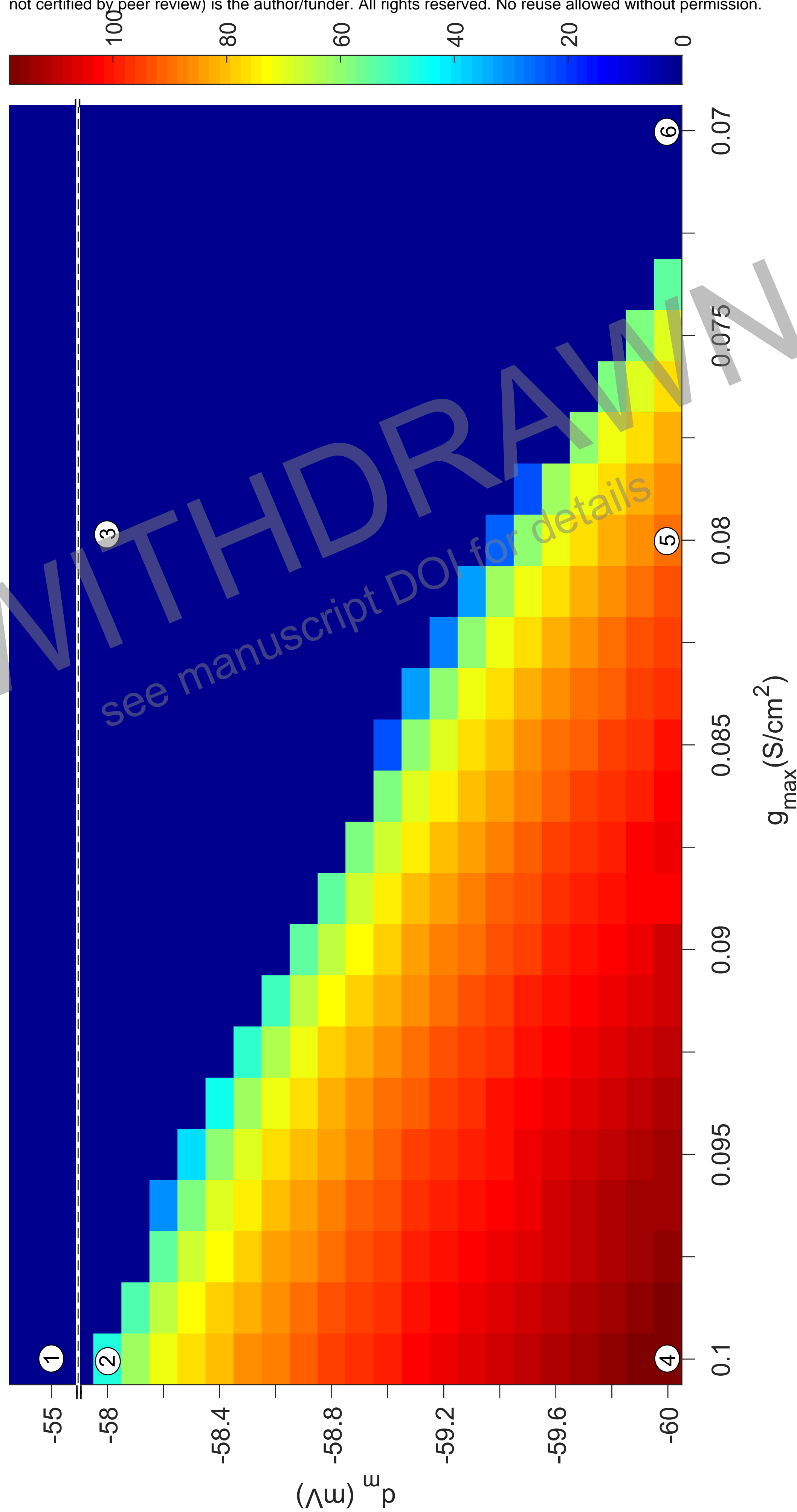

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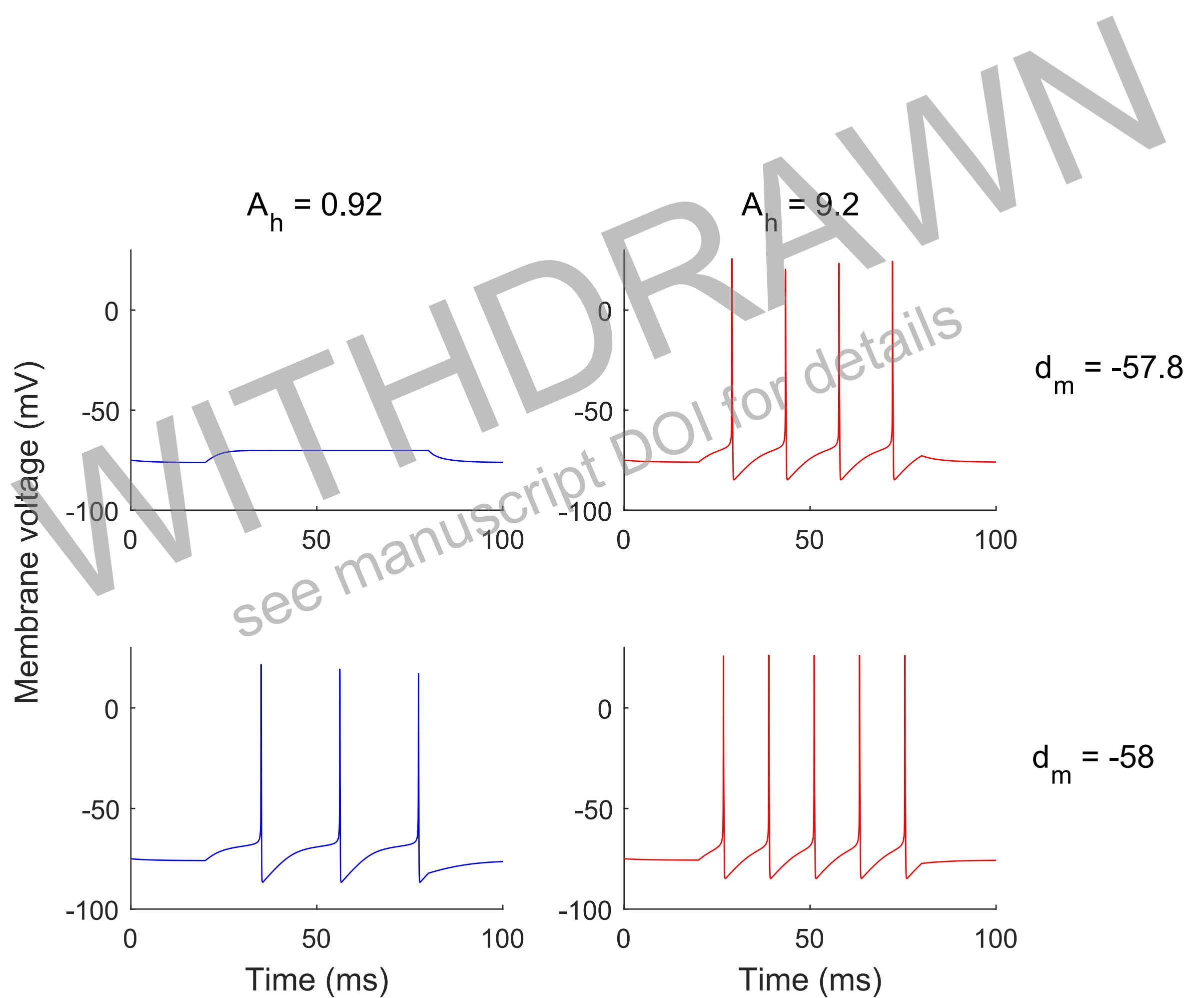

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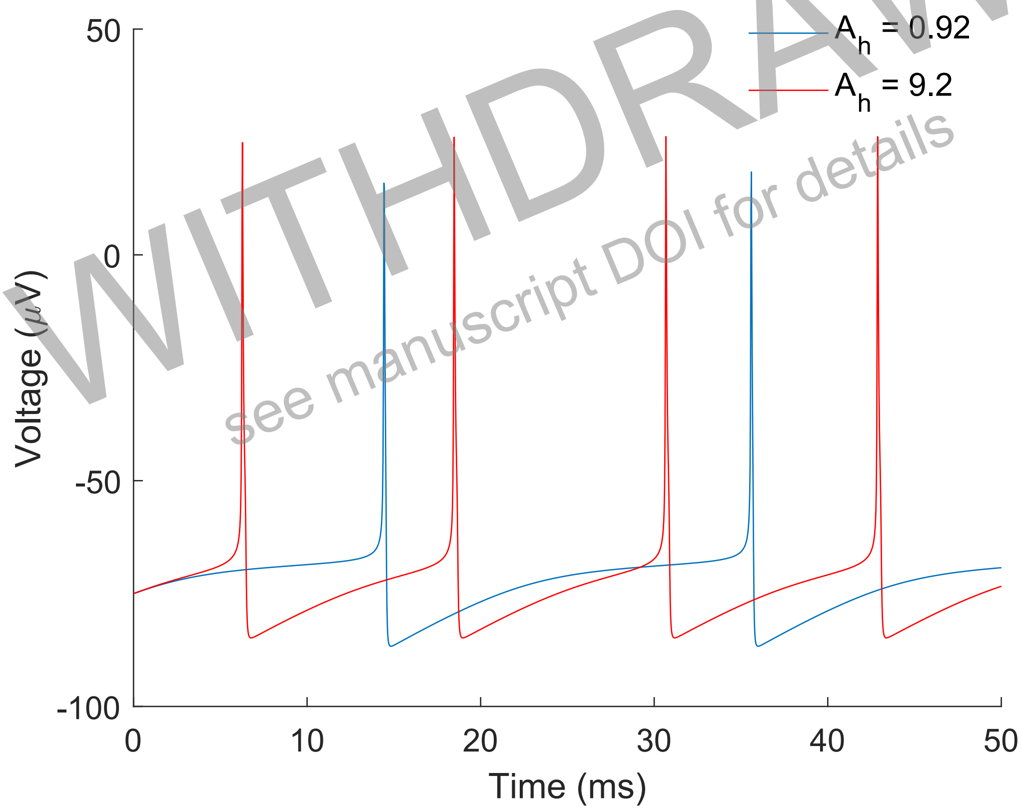

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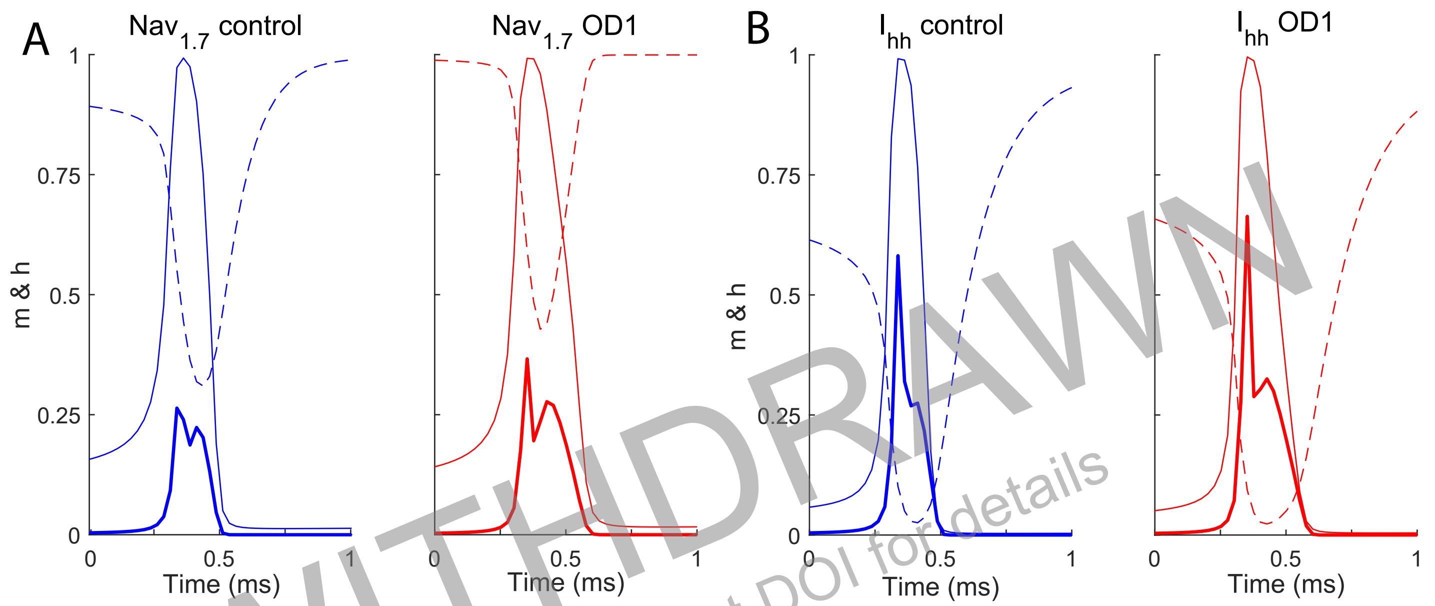

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