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1 **Direct evidence for high affinity blockade of Na_v1.6 channel subtype**
2 **by huwentoxin-IV spider peptide, using multiscale functional**
3 **approaches**

4
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18
19 Running title: Huwentoxin-IV and Na_v1.6 channel subtype

20
21 **Highlights**

- 22 • HwTx-IV inhibits *in vivo* nerve-elicited compound muscle action potential in mice
- 23 • HwTx-IV blocks *in vitro* nerve- and not directly-evoked mouse muscle
- 24 contraction
- 25 • HwTx-IV preferentially blocks TTX-S Na_v subtypes overexpressed in HEK-293
- 26 cells
- 27 • HwTx-IV is more potent to block TTX-S than TTX-R Na_v channels of mouse
- 28 DRG neurons

29
30 **Ethical statement.** All efforts were made to minimise the suffering of mice, and a
31 minimal number of animals was used. All experimental procedures were carried out
32 in accordance with the guidelines established by the French Council on animal care
33 "Guide for the Care and Use of Laboratory Animals" (EEC86/609 Council Directive –
34 Decree 2001-131), and the experimental protocols were approved on November 27
35 2015 by the French General Directorate for Research and Innovation (project
36 APAFIS#2671-2015110915123958v3 authorized to E.B.).

1 ABSTRACT

2 The Chinese bird spider huwentoxin-IV (HwTx-IV) is well-known to be a highly
3 potent blocker of Na_v1.7 subtype of voltage-gated sodium (Na_v) channels, a
4 genetically validated analgesic target, and thus promising as a potential lead
5 molecule for the development of novel pain therapeutics. In the present study, the
6 interaction between HwTx-IV and Na_v1.6 channel subtype was investigated using
7 multiscale (from *in vivo* to individual cell) functional approaches. HwTx-IV was
8 approximately 2 times more efficient than tetrodotoxin (TTX) to inhibit the
9 compound muscle action potential recorded from the mouse skeletal neuromuscular
10 system *in vivo*, and 30 times more effective to inhibit nerve-evoked than directly-
11 elicited muscle contractile force of isolated mouse hemidiaphragms. These results
12 strongly suggest that the inhibition of nerve-evoked skeletal muscle functioning,
13 produced by HwTx-IV, resulted from a toxin-induced preferential blockade of Na_v1.6,
14 compared to Na_v1.4, channel subtype. This was confirmed by whole-cell automated
15 patch-clamp experiments performed on human embryonic kidney (HEK)-293 cells
16 overexpressing hNa_v1.1-1.8 channel subtypes. HwTx-IV was also approximately
17 850 times more efficient to inhibit TTX-sensitive than TTX-resistant sodium currents
18 recorded from mouse dorsal root ganglia neurons. Finally, based on our data, we
19 predict that blockade of the Na_v1.6 channel subtype was involved in the *in vivo*
20 toxicity of HwTx-IV, although this toxicity was more than 2 times lower than that of
21 TTX. In conclusion, our results provide detailed information regarding the effects of
22 HwTx-IV and allow a better understanding of the side-effect mechanisms involved *in*
23 *vivo* and of channel subtype interactions resulting from the toxin activity.

24
25 **Keywords:** huwentoxin-IV, voltage-gated sodium channels, Na_v channel subtypes,
26 electrophysiology, mouse neuromuscular excitability, mouse dorsal root ganglia
27 neurons, cell lines overexpressing Na_v channel subtypes

1 **Abbreviations**

2	Ca _v channel	voltage-gated calcium channel
3	CHO	Chinese hamster ovary
4	CMAP	compound muscle action potential
5	DMEM	Dulbecco's modified Eagle's medium
6	DRG	dorsal root ganglia
7	ERG	<i>ether-a-go-go</i> -related gene
8	h	human
9	HEK	human embryonic kidney
10	HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
11	HwTx-IV	huwentoxin-IV
12	i.m.	intramuscular
13	IC ₅₀	toxin concentration necessary to inhibit 50% of the response
14	ICK	inhibitory cystine knot
15	K _v channel	voltage-gated potassium channel
16	NaSpTx	voltage-gated sodium channel spider toxin
17	Na _v channel	voltage-gated sodium channel
18	PBS	phosphate buffered saline
19	ProTx-II	protoxin-II
20	r	rat
21	TTX	tetrodotoxin
22	TTX-R	resistant to tetrodotoxin
23	TTX-S	sensitive to tetrodotoxin

24 1. Introduction

25

26 Pain treatment diversification remains a big health and economic concern, and
27 animal venom peptides seem to be an original source of new antinociceptive drugs
28 targeting ion channels implicated in pain signalling pathways. Their modest size,
29 compact structure due to disulfide bridges, physicochemical resistance to enzyme
30 degradation and tissue clearance make them good candidates (Saez et al., 2010). A
31 FDA-approved painkiller is already commercialized as Prialt® since a decade. This
32 peptide derived from a conus venom toxin, blocks the $Ca_v2.2$ subtype (N-type) of
33 voltage-gated calcium (Ca_v) channels to relieve chronic pain of spinal cord injury non
34 treatable by opioid therapy (Molinski et al., 2009). Unfortunately, the peptide
35 administered *via* intrathecal injection induces severe side-effects which restrain its
36 wider application (Wang et al., 2016). Even if Prialt® is still the only venom-derived
37 drug approved for pain treatment, many other toxins entered in pre-clinical or clinical
38 development due to their capacity to interact with major molecular targets of pain
39 pathways, such as sodium, calcium, and acid-sensing ion channels, as well as
40 nicotinic receptors (Netirojjanakul and Miranda, 2017).

41 Due to their major role in initiating and propagating action potentials, voltage-
42 gated sodium (Na_v) channels are implicated in numerous pain diseases. Some of
43 these diseases can be directly linked to mutations of genes encoding the $Na_v1.7$
44 channel subtype, leading to either exacerbation of pain, such as inherited
45 erythromelalgia, paroxysmal extreme pain disorder and small fiber neuropathy, or no
46 pain sensation at all, such as congenital insensitivity to pain or type-IIID hereditary
47 sensory and autonomic neuropathy (de Lera Ruiz and Kraus, 2015; Vetter et al.,
48 2016). Na_v channels are transmembrane proteins composed of a pore-forming α
49 subunit constituted by four domains, each containing 6 transmembrane segments,
50 the S1-S4 forming the voltage-sensing domain and the S5 connected to the S6 with
51 an extracellular re-entrant P-loop constituting the ion-conducting pore (Goldin et al.,
52 2000; Catterall, 2000; de Lera Ruiz and Kraus, 2015). The gating properties and
53 turnover of Na_v channels may be modified by some members of the cell adhesion
54 molecule superfamily, the β subunits, linked to the α subunit (Namadurai et al.,
55 2015).

56 More than ten toxins have been reported so far to act selectively and potently

57 on Na_v1.7 channel subtype (Vetter et al., 2016). Among these toxins, protoxin-II
58 (β/ω -theraphotoxin-Tp2a or ProTx-II) and huwentoxin-IV (μ -theraphotoxin-Hs2a or
59 HwTx-IV) have been well characterized (Xiao et al., 2014). HwTx-IV, extracted from
60 the venom of the Chinese bird spider *Cyriopagopus schmidti*, formerly known as
61 *Haplopelma schmidti* (Schmidt, 2010), is a 35 amino acid peptide, with an inhibitory
62 cystine knot (ICK) architectural motif, belonging to Na_v channel spider toxin
63 (NaSpTx) family 1 (Klint et al., 2012). HwTx-IV was first identified as a highly potent
64 blocker of the genetically validated analgesic target Na_v1.7 channel subtype,
65 showing in addition a marked selectivity for this channel subtype compared, in
66 particular, to the heart channel subtype Na_v1.5 and the skeletal muscle channel
67 subtype Na_v1.4 which were relatively resistant to the peptide (Xiao et al., 2008).
68 Great efforts were then provided to link the promising toxin to an antinociceptive
69 effect *via* Na_v channels. The first report introduced the toxin as having a high efficacy
70 on tetrodotoxin (TTX)-sensitive Na_v channels located in rat dorsal root ganglia (DRG)
71 neurons [toxin concentration necessary to inhibit 50% of the response (IC₅₀) of 30
72 nM] which are involved in pain pathway (Peng et al., 2002). More recently, HwTx-IV
73 was reported to have analgesic effects in rodent inflammatory and neuropathic pain
74 models (Liu et al., 2014a). The peptide was thus promising as a potential lead
75 molecule for the development of novel pain therapeutics. However, until now, very
76 few information was reported about the efficiency of HwTx-IV to interact with the
77 Na_v1.6 channel subtype, a TTX-sensitive (TTX-S) Na_v channel subtype present in
78 the brain, in DRG neurons and in Ranvier nodes of α -motoneurons, as well as the
79 resulting *in vivo* side-effects resulting from this interaction (Rahnama et al., 2017).
80 This point is very important since such interaction would, in particular, impair the
81 neuromuscular system functioning, leading to the death of experimental animals, as
82 previously suggested (Liu et al., 2014b; Flinspach et al., 2017; Rahnama et al., 2017;
83 Deuis et al., 2016).

84 The present study was therefore undertaken to determine the interaction
85 between HwTx-IV and Na_v1.6 channel subtype, using multiscale (from *in vivo* to
86 individual cell) functional approaches. Our results provide detailed information
87 regarding the effects of the peptide on the mouse neuromuscular system *in vivo*, on
88 isolated mouse neuromuscular preparations, on mouse DRG neurons and on human
89 embryonic kidney (HEK)-293 cells heterologously overexpressing the human (h)

90 Na_v1.6 channel subtype, therefore providing a clue to understand the mechanisms of
91 *in vivo* motor side-effects resulting from HwTx-IV and Na_v1.6 channel subtype
92 interaction.

93

94 **2. Material and methods**

95

96 *2.1. Toxins*

97 Lyophilized synthetic HwTx-IV (molecular weight of 4106.811, purity rate >
98 97%) was obtained from Smartox Biotechnology (Saint Martin d'Hères, France). This
99 toxin was dissolved in phosphate buffered saline (PBS 1X) solution to give a
100 2.5×10^{-2} g/mL (*i.e.* 6.1 mM) stock solution. TTX citrate (molecular weight of 319.27,
101 purity rate > 98%) was purchased from Sigma-Aldrich (Saint-Quentin Fallavier,
102 France). This toxin was dissolved in PBS 1X solution to give a 9×10^{-4} g/mL (*i.e.* 2.85
103 mM) stock solution. Just prior to experiments, successive dilutions were performed in
104 the adequate standard physiological medium to give the HwTx-IV and TTX final
105 concentrations indicated in the text.

106

107 *2.2. Experimental animals*

108 Adult female Swiss mice (10-12 weeks of age and 28-32 g body weight) were
109 purchased from Janvier Elevage (Le Genest-Saint-Isle, France) and housed at the
110 CEA animal facility. The experiments were performed in accordance with the
111 guidelines established by the French Council on animal care "Guide for the Care and
112 Use of Laboratory Animals" (EEC86/609 Council Directive – Decree 2001-131), on
113 mice under anaesthesia by means of isoflurane (AErrane®, Baxter S.A., Lessines,
114 Belgique) inhalation. The experimental protocols were approved on November 27
115 2015 by the French General Directorate for Research and Innovation (project
116 APAFIS#2671-2015110915123958v3 authorized to E.B.).

117

118 *2.3. Isolated mouse neuromuscular preparations*

119 Mouse hemidiaphragms with their respective associated phrenic nerve were
120 rapidly and carefully dissected after anaesthesia (by isoflurane inhalation) and
121 euthanasia (by cervical vertebrae dislocation) of mice. After removal, the isolated
122 neuromuscular preparation was mounted on a silicone-lined organ bath (4 mL

123 volume) filled with a standard physiological medium of the following composition (in
124 mM): NaCl 145, KCl 5, CaCl₂ 2, MgCl₂ 1, D-glucose 11, and N-2-
125 hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 5 (pH 7.4, adjusted with
126 NaOH), continuously gassed with pure O₂ throughout the experiment.

127

128 *2.4. Primary cultures of mouse DRG neurons*

129 Mouse DRG were removed from the animal spinal cord, placed in iced-Ham's
130 F-12 medium (Sigma-Aldrich) and enzymatically dissociated with collagenase type IA
131 (2 mg/mL; Sigma-Aldrich) and dispase (5 mg/mL; Gibco, Thermo Fisher Scientific,
132 Villebon-sur-Yvette, France). Neurons were then plated on 12-mm glass coverslips
133 placed in a 24-wells plate coated with 10 µg/mL of poly-D-lysine and 100 µg/mL of
134 murin laminin (Sigma-Aldrich). The cells were maintained in culture at 37°C (in 95%
135 air and 5% CO₂) in Neurobasal A medium (Gibco) containing horse serum (5%;
136 Gibco), penicillin/streptomycin (47.64 U/mL; Gibco), nerve growth factor (83.33
137 ng/mL; Sigma-Aldrich), N2 supplement (3.18x; Gibco), Dulbecco's phosphate-
138 buffered saline (1X) w/o CaCl₂ and MgCl₂ (1.68%; Gibco), bovine serum albumin
139 (16.83 µg/mL; Sigma-Aldrich), corticosteron (214.85 nM; Sigma-Aldrich), T3 hormone
140 (56.06 nM; Sigma-Aldrich) and L-glutamine (1.90 mM; Sigma-Aldrich). Cytosine β-D-
141 arabinofuranoside (2 µM; Sigma-Aldrich) was added to the culture medium, 24 hours
142 later, to stop astrocyte proliferation.

143 Experiments were carried out within 2 to 6 days after cell plating. The day of
144 their use, the neurons plated on coverslips were transferred, for a minimum of 30 min
145 at 37°C prior to manual patch-clamp recordings, in 35-mm Petri dishes filled with a
146 standard physiological medium of the following composition (in mM): NaCl 134, KCl
147 3, CaCl₂ 1, MgCl₂ 1, D-glucose 10, tetraethylammonium chloride (TEA) 10, CdCl₂
148 0.1, and HEPES 10 (pH 7.35, adjusted with NaOH), and then in the recording bath
149 filled with the standard physiological medium.

150

151 *2.5. HEK-293 and Chinese hamster ovary (CHO) cell line cultures*

152 HEK-293 cells heterologously overexpressing hNav_v1.7 (Eurofins, Discovery
153 Services, St Charles, MO, USA) were cultured in suspension in FreestyleTM293
154 (Gibco; #12338-018). Those overexpressing hNav_v1.1 (Eurofins), hNav_v1.6 (ChanTest,
155 Cleveland, OH, USA) or hNav_v1.8 (Millipore, Billerica, MA, USA) were cultured in

156 Dulbecco's modified Eagle's medium (DMEM)/F-12 nutrient mixture with GlutaMAX™
157 supplement (Gibco; #31331), those overexpressing hNa_v1.2 (SB Drug Discovery,
158 Glasgow, United Kingdom) and hNa_v1.5 (Sanofi, Frankfurt, Germany) in DMEM with
159 GlutaMAX™ supplement (Gibco; #31966), and those overexpressing hNa_v1.3 and
160 1.4 (SB Drug Discovery) in minimum essential medium (Sigma-Aldrich; #M5650).
161 CHO cells heterologously overexpressing hCa_v1.2/β2/α2δ1 (ChanTest, Cleveland,
162 OH, USA) were cultured in Ham's F12 nutrient mix with GlutaMAX™ medium (Gibco;
163 #31765). Those overexpressing the voltage-gated potassium (K_v) channel subtype
164 hK_v11.1 (B'SYS GmbH, Switzerland), encoded by the human *ether-a-go-go*-related
165 gene (hERG), were cultured in DMEM/F-12 nutrient mixture Ham's medium (Sigma;
166 #D6434). All culture media contained fetal bovine serum (10% v/v) and selected
167 antibiotics and additives, as recommended by the manufacturer. Cells were grown in
168 flasks, in a humidified 5% CO₂ incubator at 37°C, and subcultured/passaged every 3-
169 4 days using TrypLE Select enzyme (Gibco).

170 For manual patch-clamp recordings, a few days before experiments, the cells
171 were transferred on 12-mm glass coverslips placed in 35-mm Petri dishes. The day
172 of their use, the culture medium was changed to a standard physiological medium,
173 similar to that used for DRG neurons (see paragraph 2.4), for a minimum of 15 min at
174 37°C prior to experiments. The cells plated on coverslips were then transferred in the
175 recording bath filled with the standard physiological medium. For automated patch-
176 clamp recordings, the day of their use, the cells were transferred in Eppendorf tubes
177 containing a FreeStyle 293 expression medium (Gibco; #12338) which were then
178 placed in the automated electrophysiology platform.

179

180 2.6. Electrophysiological recordings from the mouse neuromuscular system in vivo

181 Recordings from the neuromuscular system of anaesthetized mouse were
182 obtained by means of a minimally-invasive electrophysiological method, using the
183 Qtrac© software (Prof. H. Bostock, Institute of Neurology, London, United Kingdom),
184 as previously detailed (Marrouchi et al., 2013). Briefly, the anaesthetized mice were
185 placed on a heating pad to maintain body temperature throughout the experiments
186 (between 35.94 ± 0.05 and 35.99 ± 0.03°C, as determined in 29 mice using a rectal
187 probe). Electrical stimulations were delivered to the caudal motor nerve (at the base
188 of the tail) by surface electrodes, and the compound muscle action potential (CMAP)

189 was recorded using needle-electrodes inserted into the tail muscle. To study the local
190 action of HwTx-IV and TTX, intramuscular (i.m.) injections (4- μ L maximal volume) of
191 PBS 1X solution containing various concentrations of a given toxin were delivered
192 with a 10- μ L micro-syringe at the base of mouse tail, between stimulation and ground
193 electrodes. Similar injections (4 μ L) were also done with PBS 1X solution to test an
194 eventual effect of the vehicle.

195 On-line recordings were initiated approximatively 5 min before a given
196 injection to determine the toxin and/or vehicle effects on selected excitability
197 parameters, such as the excitability threshold and CMAP amplitude continuously
198 registered as a function of time. To better identify the underlying mechanism(s) of
199 action and the duration of HwTx-IV and TTX effects, five different excitability tests
200 (stimulus-response, strength-duration and current-threshold relationships, as well as
201 threshold electrotonus and recovery cycle; detailed in Cerles et al., 2017) were
202 performed together before and at various times (from ~30 min to ~12 h) after a given
203 injection. As a whole, more than thirty parameters were determined from these five
204 excitability tests, and analyzed. Most of them provide specific and complementary
205 information on the density and functional status of ion channels, receptors and
206 pumps, as well as on the passive membrane properties of the neuromuscular system
207 (Kiernan and Bostock, 2000; Krishnan et al., 2008).

208

209 *2.7. Mechanical recordings from isolated mouse neuromuscular preparations*

210 Measurements of isometric contractile force (*i.e.* single twitch), elicited by
211 stimulating the muscle either indirectly *via* the phrenic nerve or directly, were
212 performed as previously described (Schlumberger et al., 2010). Briefly, the central
213 tendon of the hemidiaphragm was attached to an isometric force displacement
214 transducer (FT03 model; Grass Instruments, West Warwick, RI, USA) *via* an
215 adjustable stainless-steel hook, and the other tendon (at the rib side) was securely
216 anchored onto the silicone-coated bath. A mobile micrometer stage allowed adjusting
217 the muscle length to obtain maximal contractile responses. The resting tension was
218 monitored and kept constant throughout the experiment.

219 Electrical square pulses of 200 μ s duration and supramaximal voltage (15-20
220 V for nerve stimulation and 80-100 V for direct stimulation) were delivered by a
221 stimulator (S-48 model; Grass Instruments) at a frequency of 0.1 Hz. Signals from

222 the force transducer were amplified, collected, and digitized with the aid of a
223 computer equipped with an analog-to-digital converter (Digidata-1322A model;
224 Molecular Devices, Sunnyvale, CA, USA) managed by the Axoscope 9.2 software
225 (Molecular Devices). The collected signals were analyzed off-line using the Clampfit
226 9.2 program (Molecular Devices). The experiments were carried out at constant room
227 temperature (22°C).

228

229 *2.8. Automated patch-clamp recordings from HEK-293 and CHO cells*

230 Whole-cell automated patch-clamp experiments were performed on a QPatch
231 HTX automated electrophysiology platform (Sophion Bioscience, Ballerup, Denmark),
232 allowing both signal acquisition and data analyses (Bell and Dallas, 2017). The
233 extracellular medium composition of hNa_v-overexpressing HEK293 cells was (in
234 mM): NaCl 154, KCl 4, CaCl₂ 2, MgCl₂ 1, and HEPES 10 (pH 7.4, adjusted with
235 NaOH), and that of intracellular (*i.e.* patch-clamp pipette) medium: CsF 150,
236 EGTA/CsOH 1/50, HEPES 10, NaCl 10, MgCl₂ 1, and CaCl₂ 1 (pH 7.4, adjusted with
237 CsOH). The extracellular medium composition of hCa_v1.2-overexpressing CHO cells
238 was (in mM): NaCl 145, KCl 4, CaCl₂ 10, and HEPES 10 (pH 7.4, adjusted with
239 NaOH), and that of intracellular medium: CsF 27, CsCl 112, EGTA 8.2, HEPES 10,
240 NaCl 2, and Mg-ATP 4 (pH 7.4, adjusted with CsOH). The extracellular medium
241 composition of hK_v11.1-overexpressing CHO cells was (in mM): NaCl 145, KCl 4,
242 CaCl₂ 2, MgCl₂ 1, HEPES 10, and D-glucose 10 (pH 7.4, adjusted with NaOH), and
243 that of intracellular medium: KCl 120, CaCl₂ 5.4, EGTA 10, HEPES 10, Mg-ATP 4,
244 and MgCl₂ 1.75 (pH 7.4, adjusted with KOH). HwTx-IV was diluted in the extracellular
245 medium added with bovine serum albumin (0.1%), to give the final concentrations
246 indicated in the text. The times of incubation varied between ~2 and ~7 min to
247 achieve steady-state effects. The experiments were carried out at room temperature
248 (20-22°C).

249 The hNa_v-overexpressing HEK-293 cells were maintained at a holding
250 potential of either -90 mV (hNa_v1.5) or -100 mV (other hNa_v channel subtypes).
251 Currents were elicited at a frequency of 0.2 Hz by 20-ms test-pulses to -20 mV
252 (hNa_v1.1, 1.2, 1.4, and 1.7), -10 mV (hNa_v1.3), -40 mV (hNa_v1.5), -15 mV (hNa_v1.6)
253 or +10 mV (hNa_v1.8), preceded by 200-ms (hNa_v1.5) or 40-ms (hNa_v1.7) pulses to -
254 120 mV, or not (hNa_v1.1, 1.2, 1.3, 1.4 and 1.6). The hCa_v1.2-overexpressing CHO

255 cells were maintained at a holding potential of -50 mV, and currents were elicited at a
256 frequency of 0.05 Hz by 200-ms test-pulses to +0 mV. The hK_V11.1-overexpressing
257 CHO cells were maintained at a holding potential of -80 mV, and tail currents were
258 elicited at a frequency of 0.07 Hz by 5-s test-pulses to -50 mV, preceded by 4.8-s
259 pulses to +20 mV following 20-ms pulses to -50 mV. The concentration-response
260 relationships were established by expressing the peak amplitude of the sum of ten
261 cell currents recorded in the presence of a given HwTx-IV concentration relatively to
262 that of the sum of these currents recorded before toxin incubation.

263

264 *2.9. Manual patch-clamp recordings from HEK-293 cells and mouse DRG neurons*

265 Whole-cell manual patch-clamp experiments were performed by using a
266 MultiClamp 700B integrating patch-clamp amplifier and the pClamp10.6 software
267 (Molecular Devices, Sunnyvale, CA, USA), as previously described (Schlumberger et
268 al., 2014). The signals, acquired at a 4-kHz sample rate, were filtered at 2 kHz with a
269 low-pass Bessel filter and digitized with the aid of a computer equipped with an
270 analog-to-digital converter (Digidata-1440A model; Molecular Devices). The patch-
271 clamp pipettes were filled with a medium composed of (in mM): CsCl 90, CsMeSO₃
272 40, NaCl 10, MgCl₂ 2, EGTA 2, ATP-Na₂ 4, and HEPES 10 (pH 7.32, adjusted with
273 CsOH), and had ~3-MΩ resistance in the standard physiological medium. A fast
274 perfusion system allowed changing the solution (standard physiological medium
275 added or not with a given toxin concentration) around the recorded cell within a few
276 seconds. The experiments were carried out at constant room temperature (22°C).

277 The cells were maintained at a holding potential of -60 mV, and currents were
278 elicited at a frequency of 0.5 Hz by 50-ms test-pulses to -20 mV (+10 mV for
279 hNa_V1.6-overexpressing HEK-293 cells) preceded by 1-s pulses to -120 mV. The
280 concentration-response relationships were established by expressing the peak
281 current amplitude measured in the presence of a given toxin concentration relatively
282 to that before toxin application. Current-voltage relationships were obtained by
283 varying test-pulses from -80 to +10 mV in 5-mV increments, and steady-state
284 inactivation-voltage relationships by changing pre-pulses from -120 to -20 mV in 5-
285 mV increments.

286

287 *2.10. Data and statistical analyses*

288 Concentration-response relationships were established by plotting the
289 response, recorded in the presence of a given toxin (R_t) and expressed as
290 percentage of the value obtained in absence of toxin (R_c), against the toxin
291 concentration ($[toxin]$). The theoretical concentration-response curves were
292 calculated from typical sigmoid nonlinear regressions through data points (correlation
293 coefficient = r^2) according to the Hill equation (GraphPad Prism 5 or QPatch assay
294 software): $R_t / R_c = 1 / [1 + ([toxin] / IC_{50})^{n_H}]$, where n_H is the Hill number.

295 Conductance (g) was calculated according to the following equation: $g = I / (V_T$
296 $- V_{Na})$, where I is the peak current amplitude, V_T is the test-pulse voltage, and V_{Na} is
297 the equilibrium potential of Na ions. Conductance-voltage relationships were
298 established by plotting the conductance, expressed as percentage of the maximal
299 conductance (g_{max}) calculated at large positive test-pulses, as a function of test-
300 pulse voltage. The theoretical curves correspond to data point fits, according to the
301 Boltzmann equation (GraphPad Prism 5 software): $g / g_{max} = 1 - [1 / (1 + \exp ((V_T -$
302 $V_{T50\%}) / k_g))]$, where $V_{T50\%}$ is the test-pulse voltage corresponding to 50% maximal
303 conductance, and k_g is the slope of the curve.

304 Steady-state inactivation-voltage relationships were established by plotting the
305 peak current amplitude, expressed as percentage of the maximal amplitude (I_{max})
306 recorded in response to large negative pre-pulses, as a function of pre-pulse voltage
307 (V_P). The theoretical curves correspond to data point fits, according to the Boltzmann
308 equation (GraphPad Prism 5 software): $I / I_{max} = 1 / [1 + \exp ((V_P - V_{P50\%}) / k_h)]$,
309 where $V_{P50\%}$ is the pre-pulse voltage corresponding to 50% maximal peak amplitude
310 of current, and k_h is the slope of the curve.

311 Current kinetics were evaluated by calculating the two following activation and
312 inactivation parameters: (1) the time to peak (t_p), defined as the time between test-
313 pulse triggering and the peak current, and (2) the time constant (τ_h) of the current
314 decay, assuming that the current inactivation is a mono-exponential decay as a
315 function of time, *i.e.* $I(t) = I(0) e^{(-t/\tau_h)}$.

316 Data are expressed as means \pm standard deviations (S.D.) of n different
317 experiments. The statistical comparison of values was carried out using the
318 parametric two-tailed Student's t -test (either paired samples for comparison within a
319 single population or unpaired samples for comparison between two populations), the
320 2-factor ANOVA test or the nonparametric Mann-Whitney U test, according to the

321 equality of sample variances estimated with the Lilliefors test. Differences between
322 values were considered to be statistically significant at $P \leq 0.05$.

323

324 **3. Results**

325

326 *3.1. Effects of HwTx-IV and TTX on the mouse neuromuscular system in vivo*

327 On-line recordings revealed that the major effect of i.m. injections of PBS 1X
328 solutions containing various concentrations of either HwTx-IV (from 4.1 pmol/kg to
329 41.4 nmol/kg mouse) or TTX (from 3.4 pmol/kg to 166.5 nmol/kg mouse) to
330 anaesthetized mice was a marked concentration-dependent decrease of CMAP
331 amplitude. This is exemplified in Fig. 1A (insets) for CMAP recordings performed
332 before and ~10 min after injections of 41.4 nmol HwTx-IV and 166.5 nmol TTX per kg
333 of mouse. This effect occurred within various times, depending on the concentration
334 of toxin injected, ranging from ~10 to ~20 min (Fig. 1A). It is worth noting that the
335 maximal CMAP amplitude remained stable before toxin injections and during ~25 min
336 after injection of 3.4 pmol/kg mouse of TTX. Moreover, the maximal CMAP amplitude
337 measured ~30 min after injections of PBS 1X solution alone, and compared to values
338 before injections, was not significantly affected, *i.e.* $97.8 \pm 2.3\%$ ($n = 8$ mice),
339 indicating (i) that injections of the toxin vehicle had no effect on the maximal CMAP
340 amplitude and (ii) that no marked run-down of the response occurred.

341 The toxin blocking effect was quantified by establishing the concentration-
342 response curves, *i.e.* the maximal CMAP amplitude as a function of the concentration
343 of toxin injected, and determining the IC_{50} values (see for more details the Material
344 and methods section). As shown in Fig. 1B, the concentration-response curves for
345 HwTx-IV and TTX revealed IC_{50} values of 0.9 and 1.9 nmol/kg mouse, respectively. It
346 thus appears that HwTx-IV was approximately 2 times more efficient than TTX, on
347 equimolar basis, to inhibit the CMAP.

348

Figure 1 near here

349 The five different excitability tests (stimulus-response, strength-duration and
350 current-threshold relationships, as well as threshold electrotonus and recovery cycle)
351 were performed together before and ~30 min after i.m. injections of PBS 1X solution
352 containing HwTx-IV (4.1-41.4 nmol/kg mouse) or TTX (5.3-17.8 nmol/kg mouse), and
353 the derived neuromuscular excitability parameters were determined (Supplementary

354 data, Fig. S1, Fig. S2 and Table S1). With the exception of decreased maximal
355 CMAP amplitude and increased stimulus intensity required to generate a 50%
356 maximal amplitude CMAP (stimulus-response relationship; Fig.S1Aa, Fig. S2Aa and
357 Table S1), analysis of strength-duration relationship, threshold electrotonus, current-
358 threshold relationship, and recovery cycle did not reveal other HwTx-IV and TTX
359 effects since no significant modification of excitability waveforms and derived
360 parameters was detected (Fig. S1B-E, Fig. S2B-E and Table S1). The toxin-induced
361 effects were completely reversed within ~12 h after HwTx-IV (4.1-41.4 nmol/kg
362 mouse) or TTX (5.3-17.8 nmol/kg mouse) injections (Fig. S3 and Table S1).

363 It should be emphasized that injections of 41.4 and 394.3 nmol HwTx-IV per
364 kg of mouse (concentrations corresponding to 46 and 438 times the IC_{50} value,
365 respectively) caused death of 40% (2/5) and 80% (4/5) of the mice, respectively,
366 within one hour. Similarly, injections of 17.8 and 166.5 nmol TTX per kg of mouse
367 (*i.e.* 9 and 88 times the IC_{50} value, respectively) produced death of 43% (3/7) and
368 75% (6/8) of the animals, respectively, within one hour. These results indicate a more
369 than 2-time lower *in vivo* toxicity of HwTx-IV compared to TTX.

370

371 3.2. Effects of HwTx-IV on isolated mouse neuromuscular preparations

372 The evaluation of HwTx-IV effects on isolated mouse phrenic nerve-
373 hemidiaphragm preparations revealed that various toxin concentrations (from 152 nM
374 to 14 μ M) produced an inhibition of both nerve-evoked and directly-elicited muscle
375 contractile force of single twitches. This is illustrated in Fig. 2A (insets) for twitch
376 recordings performed in the absence and in the presence of 14 μ M HwTx-IV. The
377 toxin-induced blockade of nerve-evoked muscle twitch was concentration- and time-
378 dependent (Fig. 2). In particular, a complete inhibition of nerve-evoked contractile
379 response occurred within ~8 min after addition of 14 μ M HwTx-IV to the standard
380 physiological medium, as exemplified by the representative time-course of toxin
381 effect shown in Fig. 2A.

382

Figure 2 near here

383 Similarly, the toxin (14 μ M) produced a marked decrease of directly-elicited
384 muscle twitch within ~8 min (Fig. 2A). However, the maximal amplitude of directly-
385 elicited muscle twitch was much less affected by HwTx-IV concentrations from 152
386 nM to 14 μ M than the nerve-evoked twitch. In particular, when muscle twitch evoked

387 by nerve stimulation was completely blocked by 14 μM HwTx-IV (*i.e.* maximal
388 amplitude reduced to $1.2 \pm 0.9\%$ of initial values measured in the absence of toxin, n
389 = 5 muscles), a contractile response, although of low maximal amplitude (*i.e.* $16.9 \pm$
390 1.3% of initial values measured in the absence of toxin, $n = 5$ muscles), could be still
391 obtained by direct stimulation of hemidiaphragms. For comparison, an only slightly
392 higher maximal amplitude (*i.e.* $24.2 \pm 2.6\%$ of initial values measured in the absence
393 of toxin, $n = 10$ muscles) was obtained when muscle twitch was evoked by nerve
394 stimulation in the presence of 152 nM HwTx-IV (Fig. 2B). It is worth noting that no
395 decrease of twitch amplitude occurred when neuromuscular preparations were only
396 bathed with standard physiological medium for 20 min (data not shown). This
397 indicates, in particular, that there was no important run-down of twitch amplitude
398 under these experimental conditions.

399 Assuming that n_H was equal to 1, IC_{50} values of 30 and 900 nM were
400 calculated according to the Hill equation (see Material and methods section for more
401 details), for the toxin-induced decrease of maximal amplitude of nerve-evoked and
402 directly-elicited muscle twitches, respectively. HwTx-IV was therefore 30 times more
403 efficient to inhibit nerve-evoked than directly-elicited muscle contractile force of
404 isolated mouse hemidiaphragms.

405

406 *3.3. Effects of HwTx-IV and TTX on hNav1.1-1.8-overexpressing HEK-293 cells and* 407 *on CHO cells overexpressing hCav1.2 and hKv11.1 channel subtypes*

408 Whole-cell automated patch-clamp experiments performed on HEK-293 cells
409 overexpressing hNav1.1-1.8 channel subtypes revealed the following increasing
410 order for IC_{50} values obtained from the concentration-response curves of HwTx-IV
411 effects on currents flowing through the different channel subtypes (Fig. 3): hNav1.1
412 (41 ± 1 nM, $n = 4$) > hNav1.2 (44 ± 2 nM, $n = 10$) > hNav1.6 (52 ± 9 nM, $n = 3$) >
413 hNav1.7 (100 ± 6 nM, $n = 4$) > hNav1.3 (190 ± 24 nM, $n = 7$) > hNav1.4 (> 10 μM , $n =$
414 8) for TTX-S (IC_{50} values between 3 ± 2 and 78 ± 2 nM, $n = 2-5$) channel subtypes,
415 and hNav1.5 = hNav1.8 (> 10 μM , $n = 7-9$) for TTX-resistant (TTX-R; IC_{50} values of 3
416 ± 1 and > 10 μM , respectively, $n = 5$) channel subtypes. Therefore, all the TTX-R
417 channel subtypes studied were also relatively resistant to HwTx-IV while all the TTX-
418 S channel subtypes studied, with the exception of hNav1.4 channel subtype, were
419 also relatively sensitive to HwTx-IV. Additionally, HwTx-IV was ~2 times more potent

420 to block hNav_v1.6 than hNav_v1.7 channel subtypes. It is worth noting that the toxin had
421 very low affinity for hCa_v1.2 and hK_v11.1 channel subtypes overexpressed in CHO
422 cells since the IC₅₀ values obtained from the concentration-response curves of HwTx-
423 IV effects on currents flowing through these two ion channels were higher than 10 μM
424 (n = 2-4, data not shown).

425 Figure 3 near here

426 Detailed experiments, using whole-cell manual patch-clamp, displayed IC₅₀
427 values of 83.3 and 32.6 nM from the concentration-response curves of HwTx-IV
428 effects on currents flowing through hNav_v1.6 and hNav_v1.7 channel subtypes
429 overexpressed in HEK-293 cells, respectively (Fig. 4A). Under these conditions, the
430 toxin was thus approximately 2.5 times less efficient to block hNav_v1.6 than hNav_v1.7
431 channel subtypes. This HwTx-IV-induced blocking action occurred without any
432 change in steady-state inactivation- and conductance-voltage relationships of
433 hNav_v1.6 and hNav_v1.7 channel subtypes, as shown in Fig. 4B-C and Table 1 for toxin
434 concentrations of 70-100 and 20 nM, respectively, *i.e.* close to the IC₅₀ values.

435 Figure 4 and Table 1 near here

436

437 **3.4. Effects of HwTx-IV and TTX on mouse DRG neurons**

438 Before evaluating the effects of HwTx-IV on the sodium currents of DRG
439 neurons, the TTX sensitivity of these currents was first determined by perfusion of
440 cells with a standard physiological medium containing 100 nM TTX. Under this
441 condition, two types of neurons were recorded (Fig. 5A): those (67%, *i.e.* 12/18 cells)
442 having TTX-S current, *i.e.* which was blocked by the toxin to 5 ± 5% of initial peak
443 amplitude values within 1 min, and those (33%, *i.e.* 6/18 cells) having TTX-R current,
444 *i.e.* which was blocked by the toxin, perfused for ~6 min, to 83 ± 6% of initial peak
445 amplitude values but was completely inhibited by 100 μM TTX. It should be noted
446 that the activation and inactivation kinetics of TTX-R current were significantly slower
447 than those of TTX-S current (Fig. 5A and Table 2).

448 Table 2 near here

449 After washing-out TTX by perfusion of neurons with a toxin-free solution for 8-
450 10 min, the cell perfusion with standard physiological solutions containing various
451 HwTx-IV concentrations (from 5 nM to 20 μM) produced then a decrease of sodium
452 current amplitude which was dependent on the time following the perfusion, the toxin

453 concentration and the current sensitivity to TTX. In particular, the concentration-
454 response curves of HwTx-IV effects on the peak amplitude of TTX-S and TTX-R
455 currents revealed IC_{50} values of 0.13 and 110 μ M, respectively (Fig. 5B). It thus
456 appears that the toxin was approximately 850 times more efficient to inhibit TTX-S
457 than TTX-R sodium currents of mouse DRG neurons. The blocking effects of HwTx-
458 IV on the peak amplitude of TTX-S current were stationary approximately 7 and 2
459 min after perfusion of 0.005 and 1 μ M of toxin, respectively. Those on the peak
460 amplitude of TTX-R current were stationary approximately 6 min after perfusion of
461 10 to 20 μ M of toxin. The HwTx-IV effects on TTX-R current, not very noticeable,
462 were not further studied, in contrast to those on TTX-S current.

463 Figure 5 near here

464 The peak amplitude of TTX-S current, which was 9 and 3% of initial values
465 after perfusion of 1 and 10 μ M HwTx-IV, respectively, returned to $43 \pm 17\%$ of initial
466 values ($n = 9$) by perfusion of neurons with a toxin-free solution for 15-20 min. The
467 analyses of activation and inactivation kinetics of TTX-S sodium current in the
468 absence and in the presence of HwTx-IV concentrations close to the IC_{50} value (*i.e.*
469 0.1 μ M) revealed that the toxin did not affect these kinetics since τ_p and τ_h were not
470 significantly modified (Table 2). Similarly, HwTx-IV (0.1 μ M) did not produce any
471 alteration of steady-state inactivation- and conductance-voltage relationships for
472 neurons exhibiting TTX-S current (Fig. 5C and Table 1).

473

474 **4. Discussion**

475

476 The present study was undertaken to determine the interaction between
477 HwTx-IV and $Na_v1.6$ channel subtype, using multiscale (from *in vivo* to individual
478 cell) functional approaches. The results obtained can be summarized as follow: (i)
479 HwTx-IV was approximately 2 times more efficient than TTX to inhibit the CMAP
480 recorded from the mouse skeletal neuromuscular system *in vivo*, (ii) HwTx-IV was
481 approximately 30 times more effective to inhibit nerve-evoked than directly-elicited
482 muscle contractile force in isolated mouse hemidiaphragms, (iii) HwTx-IV was more
483 potent to block TTX-S ($hNa_v1.1$, 1.2, 1.3, 1.6 and 1.7, with the exception of $hNa_v1.4$)
484 than TTX-R ($hNa_v1.5$ and 1.8) Na_v channel subtypes overexpressed in HEK-293
485 cells, (iv) HwTx-IV was approximately 850 times more efficient to inhibit TTX-S than

486 TTX-R sodium currents of mouse DRG neurons, and (v) the *in vivo* toxicity of HwTx-
487 IV was more than 2 times lower than that of TTX.

488 The effects of i.m. injections of HwTx-IV and TTX on the mouse
489 neuromuscular system *in vivo* consisted in an inhibition of CMAP and an increase in
490 the stimulus intensity required to generate a 50% maximal amplitude CMAP. The
491 duration of the observed effects was less than 12 h. These results strongly suggest
492 that HwTx-IV and TTX produce a marked and reversible decrease of sodium
493 currents, *i.e.* of the density and/or functional status of "transient" Na_v channels, as
494 might be expected with toxins known to block these types of channels (Cestèle and
495 Catterall, 2000). Besides these effects, HwTx-IV and TTX did not modify other
496 excitability parameters, indicating that the two toxins do not affect the density and
497 functional status of other types and subtypes of ion channels, receptors and pumps,
498 nor the passive membrane properties of the neuromuscular system (Kiernan and
499 Bostock, 2000; Krishnan et al., 2008).

500 The HwTx-IV-induced inhibition of CMAP could be explained by an *in vivo*
501 action of this toxin on the Na_v channel subtypes identified so far in adult nerves and
502 skeletal muscles, *i.e.* the Na_v1.6 and Na_v1.4 channel subtypes, respectively
503 (Caldwell et al., 2000; de Lera Ruiz and Kraus, 2015). HwTx-IV would be
504 approximately 2 times more efficient than TTX to interact with and to block Na_v1.6
505 and/or Na_v1.4 channel subtypes. Available *in vitro* evidence indicated however that
506 the Na_v1.4 channel subtype is not preferentially sensitive to HwTx-IV since the toxin
507 affinity for this channel subtype ($IC_{50} > 10 \mu\text{M}$; Xiao et al., 2008) was more than 1000
508 times less than that of TTX ($IC_{50} \approx 8 \text{ nM}$; Tsukamoto et al., 2017), without giving
509 information on the interaction between HwTx-IV and Na_v1.6 channel subtype. It was
510 therefore probable that the relative high efficiency of HwTx-IV to inhibit *in vivo* the
511 CMAP, compared to TTX, results from toxin-induced preferential blockade of the
512 Na_v1.6 and not Na_v1.4 channel subtype. This hypothesis was tested directly by
513 studying the HwTx-IV effects on isolated mouse neuromuscular preparations and
514 recording the isometric contractile force elicited by stimulating either directly the
515 muscle or indirectly *via* its associated motor nerve.

516 The study of HwTx-IV effects on isolated mouse phrenic nerve-hemidiaphragm
517 preparations reveals that the toxin produced an inhibition of the nerve-evoked
518 contractile force with a calculated IC_{50} value of 30 nM. These results confirm those

519 obtained *in vivo* on the CMAP recorded from tail muscle in response to caudal motor
520 nerve stimulation, *i.e.* the relative high affinity of HwTx-IV for Na_V1.6 and/or Na_V1.4
521 channel subtypes. It is worth noting that HwTx-IV was previously reported to
522 completely block the conduction of isolated mouse phrenic nerve-hemidiaphragm
523 preparations, by acting on multiple ion channel proteins, especially the Ca_V channels
524 (Yu et al., 2014). However, this is not supported by the present results showing a
525 very low affinity of the peptide for hCa_V1.2 channel subtype overexpressed in CHO
526 cells.

527 Additionally, HwTx-IV also inhibited the directly-elicited muscle twitch of
528 isolated mouse hemidiaphragms with a calculated IC₅₀ value of 300 nM. The
529 conditions of direct muscle stimulation are very interesting because it makes possible
530 to decrease the implication of Na_V1.6 channel subtype (located in nerves and not in
531 muscles; Caldwell et al., 2000) in the contractile response, and thus to get direct
532 information on the affinity of interaction between HwTx-IV and Na_V1.4 channel
533 subtype (located in skeletal muscles and not in nerves; de Lera Ruiz and Kraus,
534 2015). The results obtained under these conditions therefore strongly suggest that
535 the toxin was 30 times less potent to block the Na_V1.4 than the Na_V1.6 channel
536 subtype. These results confirm the hypothesis that the HwTx-IV-induced inhibition of
537 nerve-evoked hemidiaphragm twitch and of the CMAP recorded *in vivo* from tail
538 muscle in response to caudal motor nerve stimulation resulted from a preferential
539 blockade of Na_V1.6 channel subtype by the toxin.

540 The following step was thus to undertake the study of HwTx-IV effects on
541 HEK-293 cells overexpressing hNa_V1.1-1.8 channel subtypes to get complementary
542 information on the relative selectivity of interaction between the toxin and Na_V1.6
543 channel subtype, as well as on the relative IC₅₀ value of HwTx-IV on this channel
544 subtype, since poor evidence was available in the literature (Rahnama et al., 2017).

545 The results obtained from whole-cell automated patch-clamp experiments
546 performed on HEK-293 cells overexpressing hNa_V1.1-1.8 channel subtypes
547 highlighted two groups of channel subtypes: (i) a relatively HwTx-IV-sensitive group
548 (IC₅₀ = 41-190 nM) including, by increasing order of IC₅₀ values, hNa_V1.1 > hNa_V1.2
549 > hNa_V1.6 > hNa_V1.7 > hNa_V1.3, and (ii) a relatively HwTx-IV-resistant group (IC₅₀ >
550 10 μM) composed of hNa_V1.4 ≈ hNa_V1.5 ≈ hNa_V1.8. These results are similar to the
551 following sequence of peptide affinities reported so far in the literature, with respect

552 to the Na_V channel subtype overexpressed in HEK-293 cells: hNa_V1.7 (IC₅₀ = 26 nM)
553 > rNa_V1.2 (IC₅₀ = 150 nM) > rNa_V1.3 (IC₅₀ = 338 nM) >> rNa_V1.4 ≈ hNa_V1.5 (IC₅₀ > 10
554 μM) (Xiao et al., 2008). The slight IC₅₀ differences between the two studies may
555 reflect those between the species, human or rat (r), of the Na_V channel subtypes
556 studied. It seems therefore that the selectivity of HwTx-IV towards hNa_V1.7 is not as
557 important as previously reported since the toxin is also highly potent to block
558 hNa_V1.1, h(r)Na_V1.2, h(r)Na_V1.3 and hNa_V1.6 channel subtypes. However, the
559 discovery that Na_V1.1 and 1.3 channel subtypes are involved in pain pathways (Chen
560 et al., 2014; Osteen et al., 2016; Cardoso and Lewis, 2017) and that Na_V1.2 channel
561 subtype is only located in the central nervous system (see de Lera Ruiz and Kraus,
562 2015) does not impair further development of HwTx-IV as a potential antinociceptive
563 agent to access only peripheral Na_V channel subtypes. In contrast, the fact that this
564 toxin also targets Na_V1.6 channel subtype is more problematic because of the
565 resulting side-effects which limit its *in vivo* efficacy in pre-clinical pain models.

566 The poor selectivity of HwTx-IV for hNa_V1.7 over hNa_V1.6 channel subtypes is
567 further supported by the recent characterization of the effects of a triple-mutant of
568 HwTx-IV (E1G, E4G and Y33W; m3-HwTx-IV) on either HEK-293 cells
569 overexpressing hNa_V1.1-1.5 channel subtypes, and the β1 auxiliary subunit, or CHO
570 cells overexpressing hNa_V1.6-1.7 and hNa_V1.8/β3 channel subtypes (Rahnama et al.,
571 2017). Despite the fact that mutations may influence the peptide selectivity over Na_V
572 channel subtypes, this study revealed quite similar Na_V channel subtype selectivity,
573 but higher blockade potency of the recombinant peptide, compared to HwTx-IV
574 (present results): hNa_V1.7 > hNa_V1.6 > hNa_V1.3 > hNa_V1.1 > hNa_V1.2 (IC₅₀ = 3.3-
575 11.9 nM) >> hNa_V1.4 (IC₅₀ = 369 nM) >>> hNa_V1.5 ≈ hNa_V1.8 (IC₅₀ > 1 μM). Taken
576 altogether, these data indicate that all the TTX-R channel subtypes studied (TTX IC₅₀
577 = 3-10 μM) were also resistant to HwTx-IV and m3-HwTx-IV blockade, while all the
578 TTX-S channel subtypes studied (TTX IC₅₀ = 3-78 nM), with the exception of hNa_V1.4
579 channel subtype, were also sensitive to HwTx-IV and m3-HwTx-IV inhibition.

580 The effects of HwTx-IV were further investigated on TTX-S and TTX-R Na_V
581 channel subtypes of adult mouse DRG neurons, *i.e.* mainly the TTX-S Na_V1.1, 1.6
582 and 1.7 channel subtypes and the TTX-R Na_V1.8 and 1.9 channel subtypes (Rush et
583 al., 2007). This study was motivated, on one hand, by the well-known important role
584 of DRG neurons as the primary sensitive pathway of chronic pain and, on the other

585 hand, by the absence of report of such study in mouse, until now. The effects
586 consisted in a marked and reversible decrease of the peak amplitude of TTX-S
587 sodium current ($IC_{50} = 0.13 \mu M$), without any significant modification of activation and
588 inactivation kinetics and voltage-dependencies. As expected from the results
589 obtained from whole-cell automated patch-clamp experiments, the peptide was
590 approximately 850 times less efficient in inhibiting the peak amplitude of TTX-R
591 sodium current ($IC_{50} = 110 \mu M$). From this point of view, HwTx-IV is very interesting
592 compared to TTX which was only approximately 90 times more potent to block TTX-
593 S than TTX-R Na_V channel subtypes (IC_{50} values of 5.5 and 500 nM, respectively).

594 These results are consistent with previous observations on adult rat DRG
595 neurons showing that 0.1 μM HwTx-IV inhibited TTX-S sodium current, without any
596 alteration of activation and inactivation kinetics, but had no effect on TTX-R currents
597 (Peng et al., 2002). Interestingly, the activation and inactivation kinetics of TTX-R
598 sodium current were slower than those of TTX-S sodium current. This is due to the
599 prevalence of TTX-R $Na_V1.8$ and to a less extent of $Na_V1.9$ channel subtypes which
600 are exceptionally characterized by slow ($Na_V1.8$) and even very slow ($Na_V1.9$)
601 activation and inactivation kinetics (Rush et al., 2007).

602 During experiments aimed to study the effects of i.m. injections of HwTx-IV
603 and TTX on the mouse neuromuscular system *in vivo*, 17.8 and 166.5 nmol TTX per
604 kg of mouse (corresponding to 9 and 88 times the *in vivo* IC_{50} value, respectively)
605 produced death of 43% and 75% of the animals, respectively, within one hour. These
606 values are close to those reported in the literature, *i.e.* i.m. injections of 5.3 μg (16.6
607 nmol) TTX per kg rodent caused the death of 50% of the animals (Xu et al., 2003).
608 By comparison, i.m. injections of HwTx-IV concentrations corresponding to 46 and
609 438 times the *in vivo* IC_{50} value produced death of 40% and 80% of the mice,
610 respectively, within one hour. Although these results indicate a more than 2-time
611 lower *in vivo* toxicity of HwTx-IV compared to TTX, the resulting side-effects of the
612 peptide may limit its use as a potential lead molecule for the development of novel
613 pain therapeutics. In particular, the therapeutic window of HwTx-IV as an analgesic
614 appears to be markedly reduced taking into account that the peptide was previously
615 reported to have analgesic activity at 100 $\mu g/kg$ mouse (*i.e.* 24 nmol/kg mouse) when
616 i.m. injected to animals (Liu et al., 2014a).

617 Since HwTx-IV had very low affinity for $hNa_V1.5$ channel subtype, as well as

618 for hCa_v1.2 and hK_v11.1 channel (IC₅₀ > 10 μM), it is unlikely that the death of mice
619 resulted from heart dysfunctioning. In contrast, given the poor selectivity of the
620 peptide over Na_v1.6 channel subtype, it is likely that impaired motor functioning is
621 involved since peptides interacting with this subtype of Na_v channels are well-known
622 to typically lead to such severe side-effects.

623

624 **5. Conclusion**

625

626 To the best of our knowledge, this is the first report showing directly that high
627 affinity interaction occurs between HwTx-IV and Na_v1.6 channel subtype. Indeed, the
628 toxin has been reported so far in the literature as being highly specific for the Na_v1.7
629 channel subtype, without effects on the Na_v1.6 channel subtype being evidenced.
630 The present study showing quite similar affinities of HwTx-IV for Na_v1.6 and Na_v1.7
631 channel subtypes allows better understanding the mechanisms of motor *in vivo* side-
632 effects produced by this peptide, and makes this molecule significantly less attractive
633 for an eventual therapeutic use as antinociceptive agent.

634

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643

644 **Conflict of interest**

645 The authors declare that they have no actual or potential conflict of interest
646 including any financial, personal or other relationships with other people or
647 organizations within three years of beginning this work.

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- 769

770 **Table 1.** Parameters of steady-state inactivation- ($V_{P50\%}$ and k_h) and conductance- ($V_{T50\%}$ and
 771 k_g) voltage relationships for HEK-293 cells overexpressing hNa_v1.6 and hNa_v1.7 channel
 772 subtypes (means \pm S.D. of 4-5 cells) and mouse DRG neurons having TTX-S sodium current
 773 (means \pm S.D. of 4 neurons).

	$V_{P50\%}$ (mV)	k_h (mV^{-1})	$V_{T50\%}$ (mV)	k_g (mV^{-1})
hNa _v 1.6 current before HwTx-IV perfusion	-61 ± 3	5.7 ± 0.3	-20 ± 2	5.4 ± 0.4
hNa _v 1.6 current after HwTx-IV perfusion ¹	-65 ± 2	5.9 ± 0.4	-22 ± 3	4.8 ± 0.4
hNa _v 1.7 current before HwTx-IV perfusion	-76 ± 3	6.9 ± 0.3	-21 ± 2	5.6 ± 0.5
hNa _v 1.7 current after HwTx-IV perfusion ²	-79 ± 3	7.4 ± 0.6	-22 ± 2	6.6 ± 0.5
TTX-S current before HwTx-IV perfusion	-80 ± 2	7.0 ± 0.6	-38 ± 4	3.7 ± 1.4
TTX-S current after HwTx-IV perfusion ³	-86 ± 7	6.3 ± 0.5	-38 ± 3	4.4 ± 1.7

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 776 ¹ 70-100 nM, ² 20 nM, and ³ 0.1 μ M.

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Table 2. Kinetics parameters of current activation (τ_p) and inactivation (τ_h) calculated from TTX-S and TTX-R sodium currents recorded from DRG neurons under the indicated conditions (means \pm S.D. of 4-12 cells).

	τ_p (ms)	τ_h (ms)
TTX-S current before TTX perfusion ¹	1.22 ± 0.29	0.78 ± 0.40
TTX-S current after TTX wash-out	0.81 ± 0.04	0.63 ± 0.18
TTX-S current after HwTx-IV perfusion ²	0.75 ± 0.06	0.60 ± 0.12
TTX-R current before TTX perfusion	$2.31 \pm 0.62^*$	$1.11 \pm 0.24^*$

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 787 ¹ 100 nM, ² 0.1 μ M. *: $P \leq 0.032$ (versus TTX-S current before TTX
 788 perfusion).
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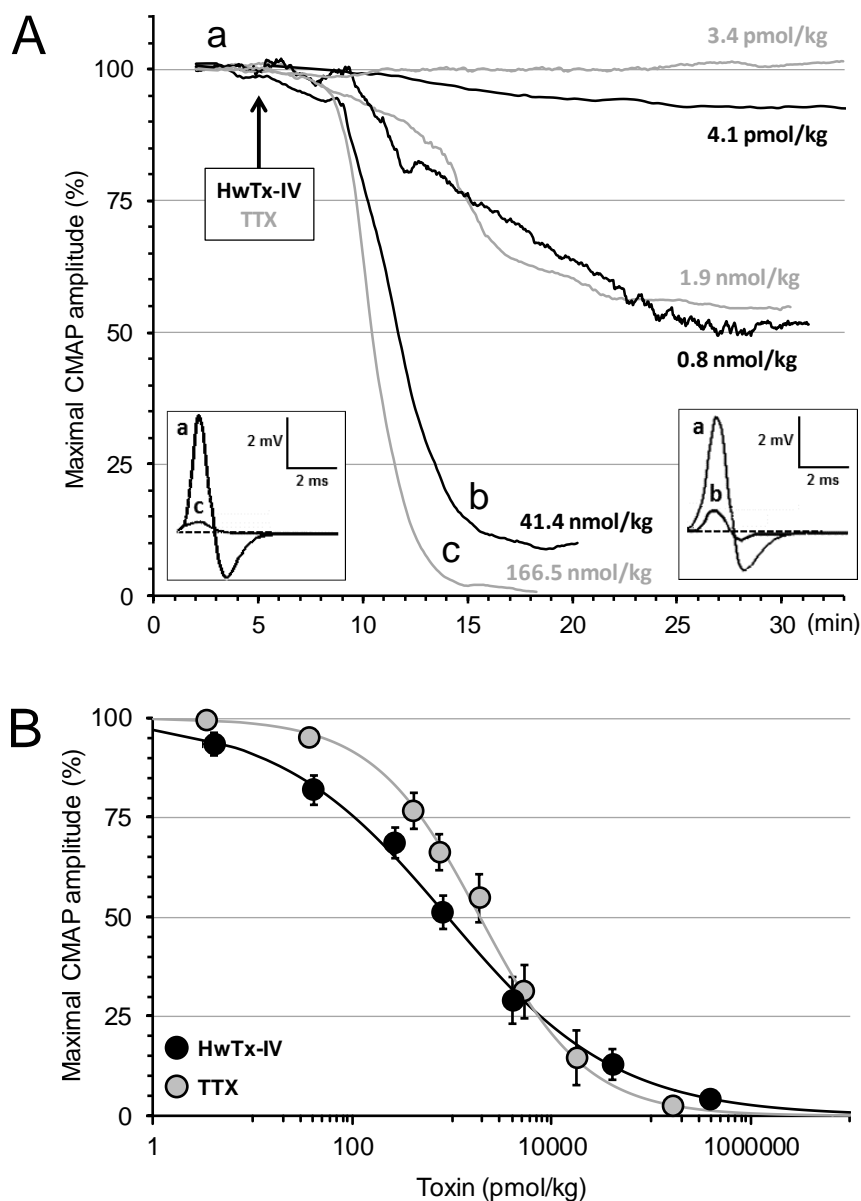


Fig. 1. Effects of HwTx-IV and TTX on the maximal CMAP amplitude recorded *in vivo* from mouse tail muscle in response to caudal motor nerve stimulation. **A:** Representative time-course of the effects of HwTx-IV (from 4.1 pmol/kg to 41.4 nmol/kg mouse, in black) and TTX (from 3.4 pmol/kg to 166.5 nmol/kg mouse, in grey) on the maximal CMAP amplitude recorded continuously as a function of time. Values are expressed as percentage of those before injections (indicated by the arrow). **Insets:** Superimposed traces of maximal CMAP, before (a) and ~10 min after HwTx-IV (41.4 nmol/kg mouse, b) or TTX (166.5 nmol/kg mouse, c) injection. **B:** Concentration-response curves of the effects of HwTx-IV (black circles) and TTX (grey circles) on the maximal CMAP amplitude. Each value, expressed as percentage of that obtained before injection, represents the mean \pm S.D. of data obtained from 4-8 mice. IC_{50} and n_H values were, respectively, 0.9 nmol/kg mouse and 0.5 for HwTx-IV ($r^2 = 0.998$) and 1.9 nmol/kg mouse and 0.8 for TTX ($r^2 = 0.997$).

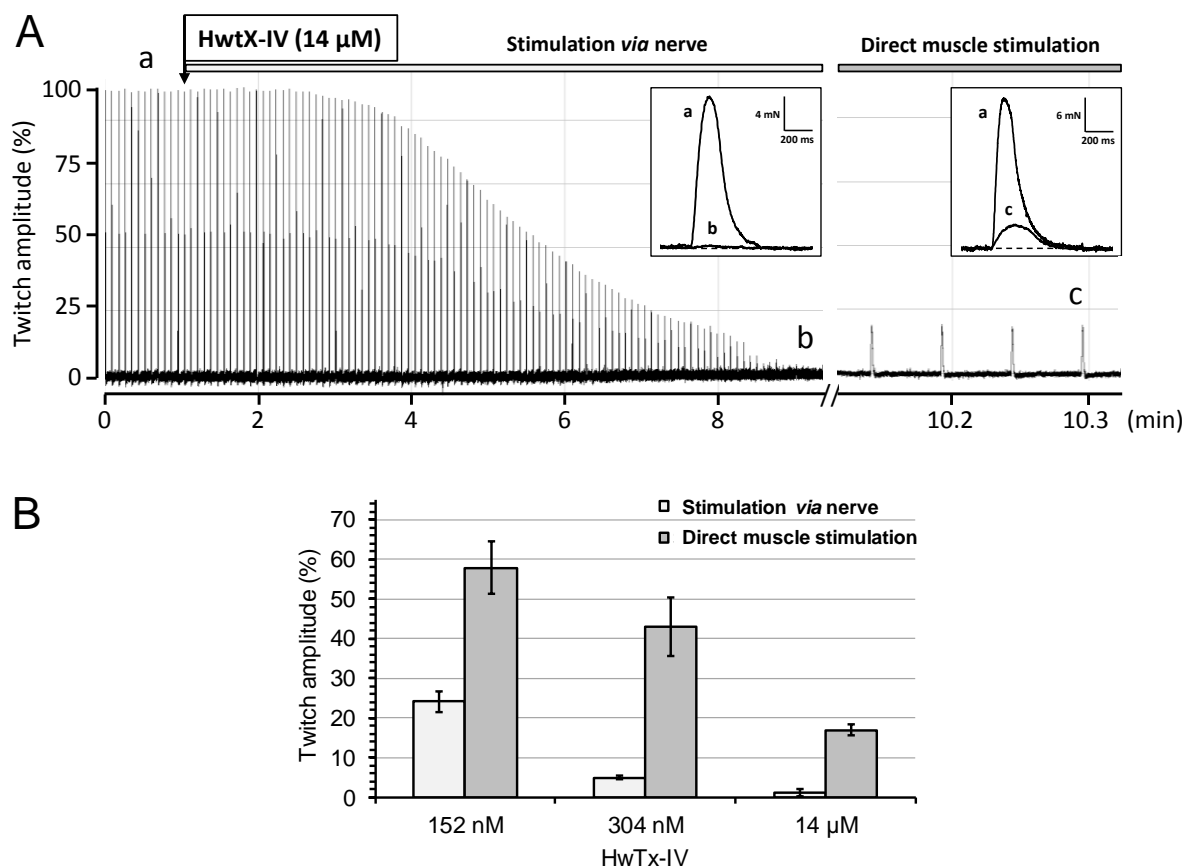


Fig. 2. Effects of HwtX-IV on nerve-evoked and directly-elicited muscle contractile force (single twitch) of isolated mouse phrenic nerve-hemidiaphragm preparation. **A:** Representative time-course of HwtX-IV (14 μ M) effects on the maximal twitch amplitude recorded continuously as a function of time, and expressed as percentage of that before toxin addition to the standard physiological medium (indicated by the arrow), in response to stimulation applied either indirectly *via* the motor nerve (light grey) or directly to muscle (dark grey). **Insets:** Superimposed traces of maximal twitch recorded before (**a**) and ~10 min after HwtX-IV (14 μ M) addition to the standard physiological medium (**b** and **c**), and elicited *via* nerve stimulation (**b**) or by direct muscle stimulation (**c**). **B:** Histogram representing nerve-evoked (light grey) and directly-elicited muscle (dark grey) maximal twitch amplitude determined in the presence of indicated HwtX-IV concentrations. Each value, expressed as percentage of that obtained before toxin addition to the standard physiological medium, represents the mean \pm S.D. of data obtained from 4-10 muscles.

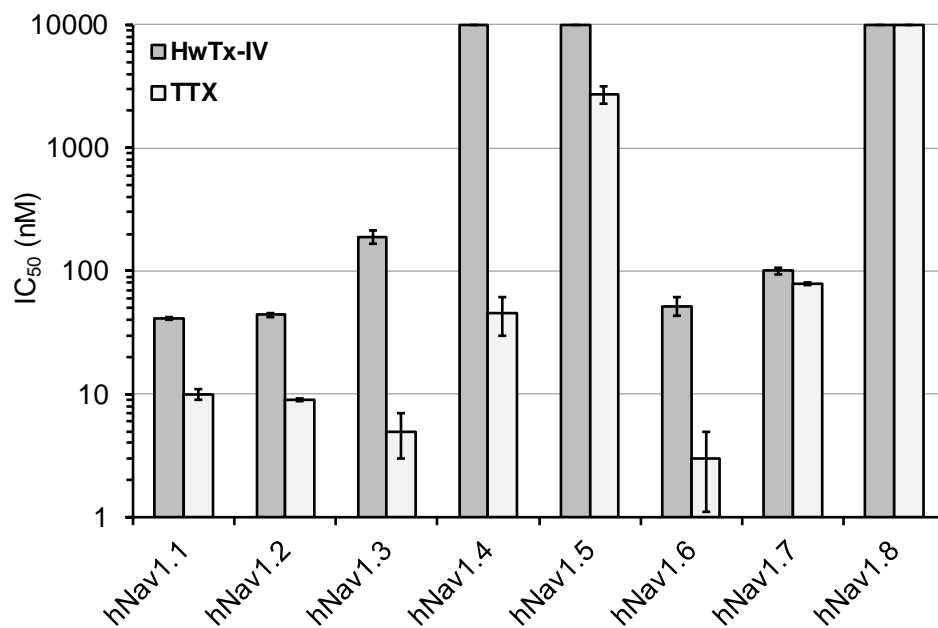


Fig. 3. Effects of HwTx-IV and TTX on HEK-293 cells overexpressing hNav_v1.1-1.8 channel subtypes. Histogram of IC₅₀ values obtained from the concentration-response curves of HwTx-IV (dark grey) and TTX (light grey) effects on hNav_v1.1-1.8 currents. Each value represents the mean \pm S.D. of data obtained from 2-10 cells. Mean values \pm S.D. of n_H were 1.3 ± 0.2 and 1.0 ± 0.1 for HwTx-IV and TTX effects, respectively.

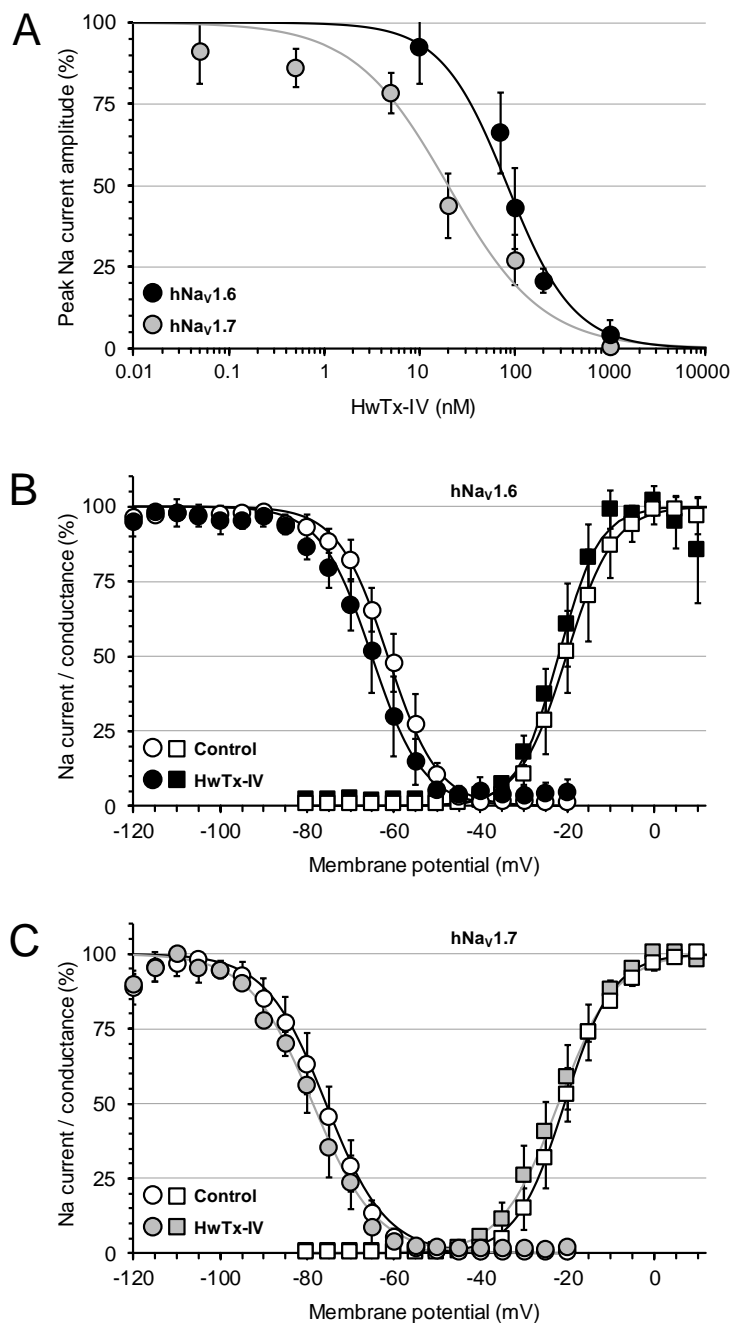


Fig. 4. Effects of HwTx-IV on HEK-293 cells overexpressing hNav_v1.6 and hNav_v1.7 channel subtypes. **A:** Concentration-response curves of HwTx-IV effects on hNav_v1.6 (black circles) and hNav_v1.7 (grey circles) currents. Each value, expressed as percentage of that obtained before toxin perfusion, represents the mean \pm S.D. of data obtained from 4-9 cells. IC₅₀ and n_H values were, respectively, 83.3 nM and 1.2 for hNav_v1.6 current ($r^2 = 0.983$), and 32.6 nM and 0.8 for hNav_v1.7 current ($r^2 = 0.901$). **B** and **C:** Steady-state inactivation- (circles) and conductance- (squares) voltage relationships for HEK-293 cells overexpressing hNav_v1.6 (**B**) and hNav_v1.7 (**C**) channel subtypes, before (open symbols) and after (close symbols) perfusion of 70-100 nM (**B**) or 20 nM (**C**) HwTx-IV. Each value represents the mean \pm S.D. of data obtained from 4-5 cells, and is expressed as percentage of either maximal peak amplitude of current at large negative pre-pulse voltages or maximal conductance calculated at large positive test-pulse voltages. The theoretical curves correspond to data point fits with the mean $V_{P50\%}$, k_h , $V_{T50\%}$ and k_g values indicated in Table 1.

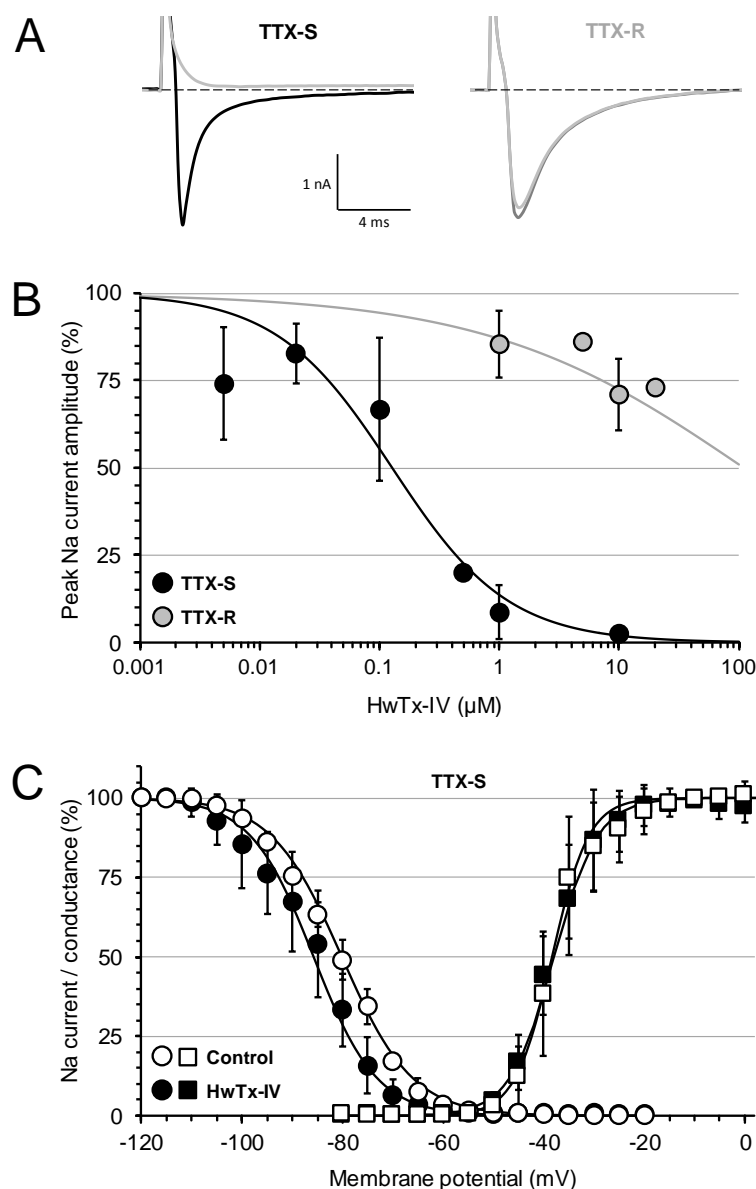


Fig. 5. Effects of HwTx-IV on TTX-S and TTX-R sodium currents of mouse DRG neurons. **A:** Representative traces of sodium currents recorded from two different neurons having either TTX-S (left) or TTX-R (right) current. In each case, the current was recorded before (black or dark grey) and 1 to 5 min after (light grey) perfusion of 100 nM TTX. **B:** Concentration-response curves of HwTx-IV effects on the peak amplitude of TTX-S (black circles) and TTX-R (grey circles) sodium currents. Each value, expressed as percentage of that obtained before toxin perfusion, represents the mean \pm S.D. of data obtained from 2-9 neurons. IC_{50} and n_H values were, respectively, 0.13 μM and 0.9 for TTX-S current ($r^2 = 0.967$), and 110 μM and 0.4 for TTX-R current ($r^2 = 0.612$). **C:** Steady-state inactivation- (circles) and conductance- (squares) voltage relationships for neurons having TTX-S current, before (open symbols) and after (close symbols) perfusion of 0.1 μM HwTx-IV. Each value represents the mean \pm S.D. of data obtained from 4 neurons, and is expressed as percentage of either maximal peak amplitude of current at large negative pre-pulse voltages or maximal conductance calculated at large positive test-pulse voltages. The theoretical curves correspond to data point fits with the mean $V_{P50\%}$, k_h , $V_{T50\%}$ and k_g values indicated in Table 1.

Direct evidence for high affinity blockade of NaV1.6 channel subtype by huwentoxin-IV spider peptide, using multiscale functional approaches

T.C. Gonçalves, R. Boukaiba, J. Molgó, M. Amar, M. Partiseti, D. Servent, and E. Benoit

SUPPLEMENTARY FIGURE 1

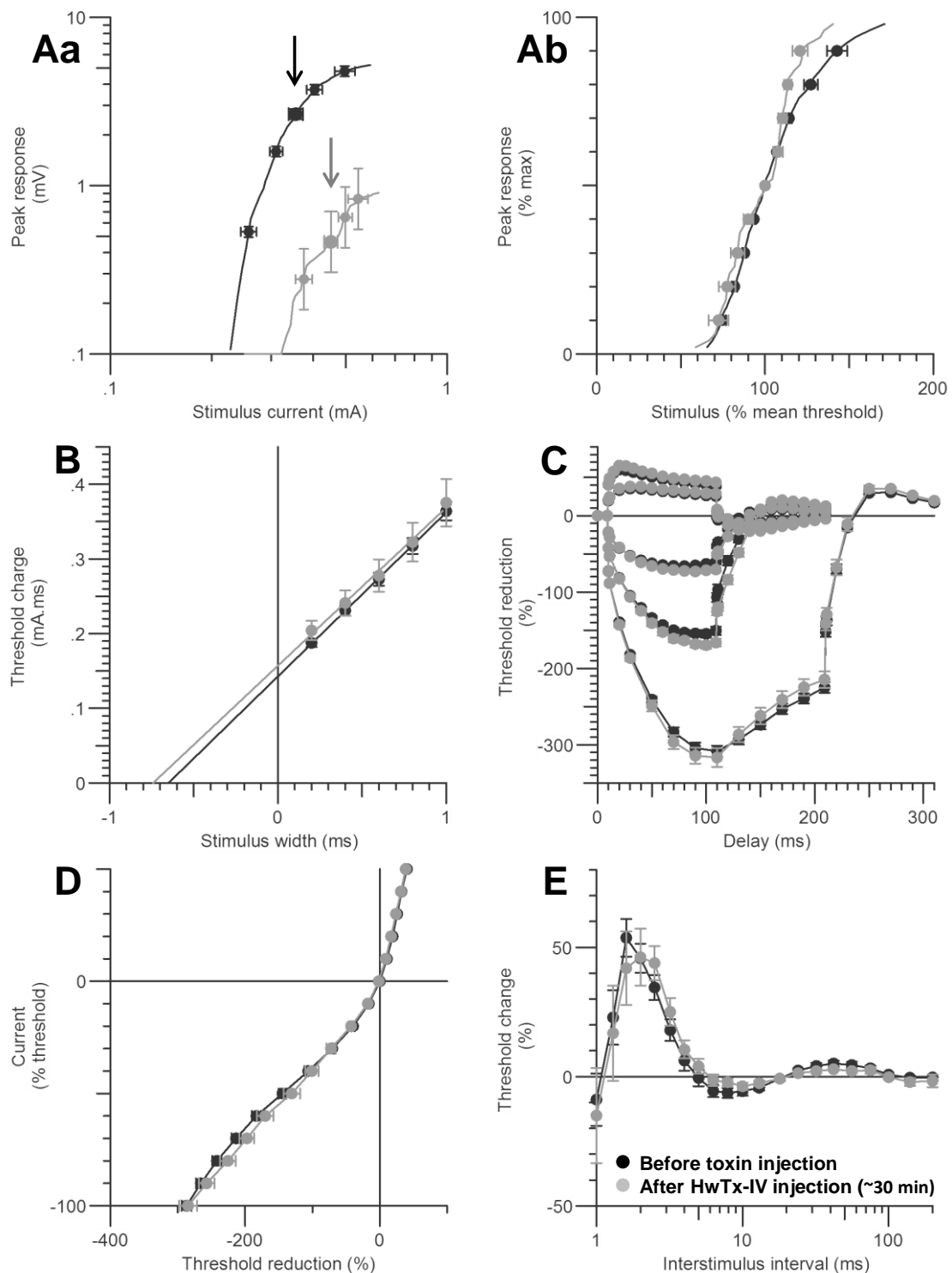


Figure S1. Superimposed excitability curves obtained *in vivo* by stimulating the mouse caudal motor nerve and recording the CMAP from tail muscle before (black circles, $n = 29$ mice) and ~ 30 min after injections of HwTx-IV (4.1-41.4 nmol/kg mouse, grey circles, $n = 6$ mice). Data are represented as means \pm S.D. **A:** stimulus-response relationships [absolute (**a**) and relative (**b**) CMAP amplitudes], **B:** strength-duration relationship, **C:** threshold electrotonus, **D:** current-threshold relationship, and **E:** recovery cycle. In **Aa**, arrows indicate stimulus currents for 50% maximal response.

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SUPPLEMENTARY FIGURE 2

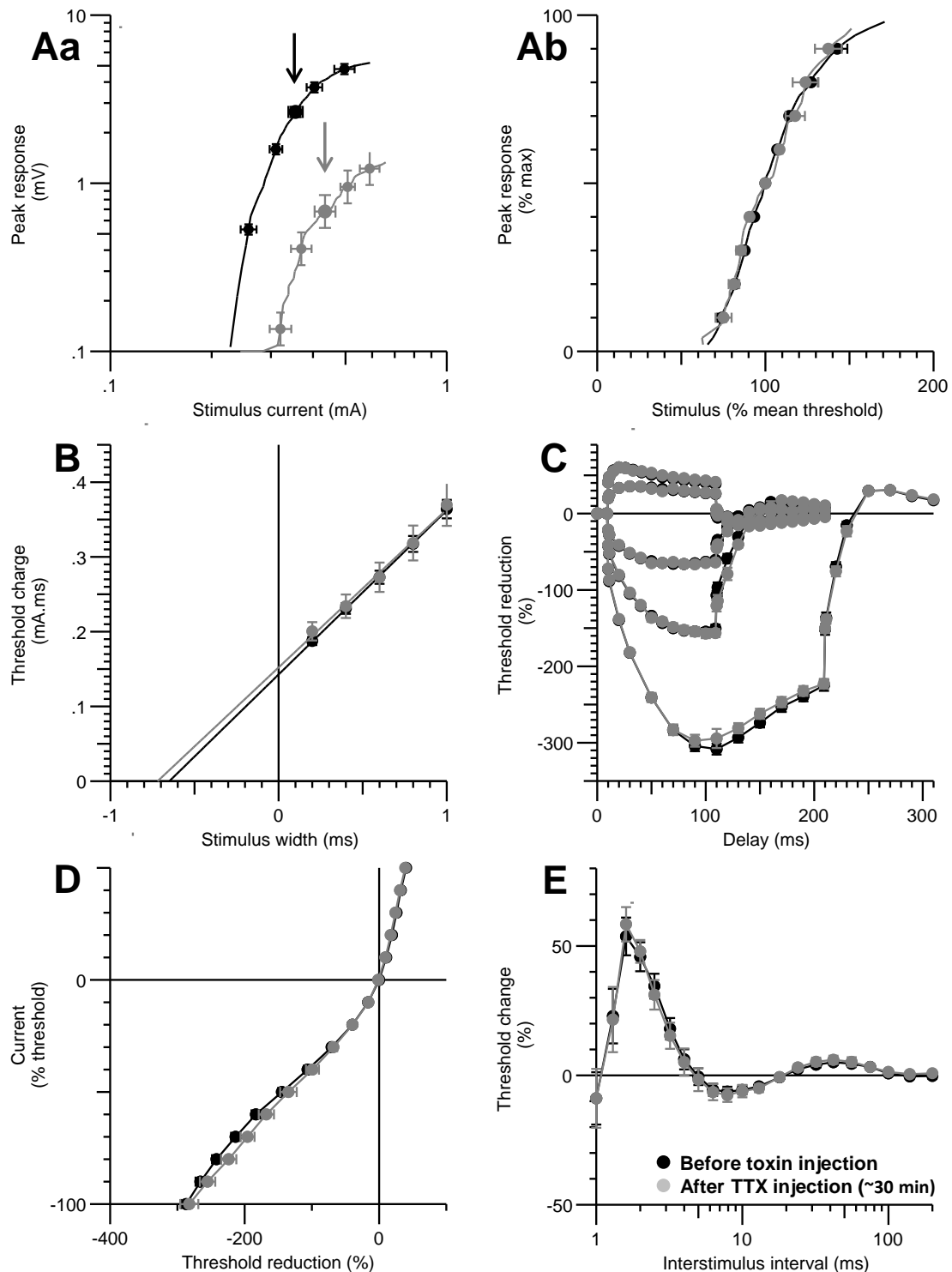


Figure S2. Superimposed excitability curves obtained *in vivo* by stimulating the mouse caudal motor nerve and recording the CMAP from tail muscle before (black circles, $n = 29$ mice) and ~ 30 min after injections of TTX (5.3-17.8 nmol/kg mouse, grey circles, $n = 9$ mice). Data are represented as means \pm S.D. **A:** stimulus-response relationships [absolute (a) and relative (b) CMAP amplitudes], **B:** strength-duration relationship, **C:** threshold electrotonus, **D:** current-threshold relationship, and **E:** recovery cycle. In Aa, arrows indicate stimulus currents for 50% maximal response.

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SUPPLEMENTARY FIGURE 3

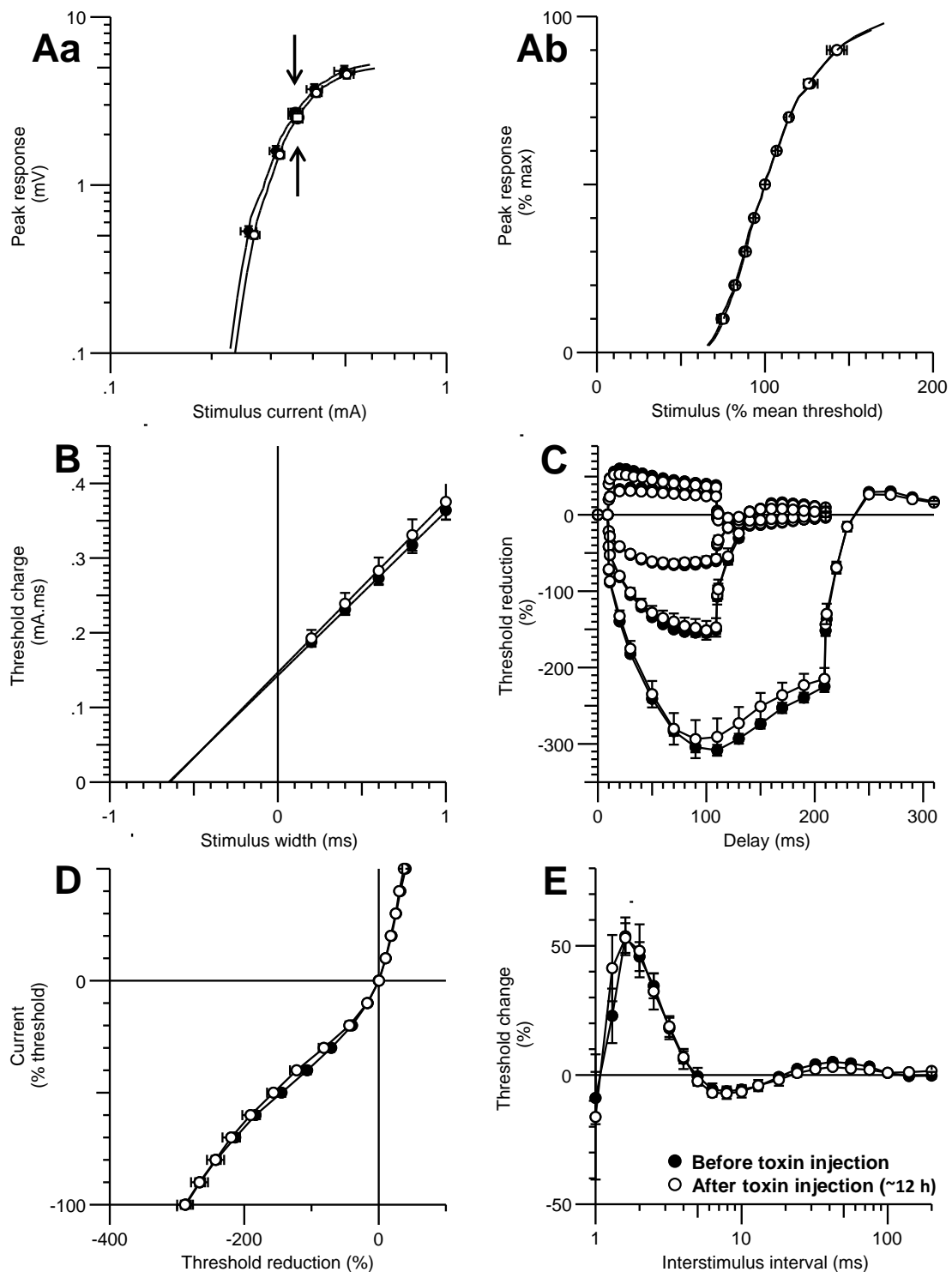


Figure S3. Superimposed excitability curves obtained *in vivo* by stimulating the mouse caudal motor nerve and recording the CMAP from tail muscle before (black circles, $n = 29$ mice) and ~ 12 h after injections (white circles, $n = 12$ mice) of HwTx-IV (4.1-41.4 nmol/kg mouse) or TTX (5.3-17.8 nmol/kg mouse). Data are represented as means \pm S.D. **A**: stimulus-response relationships [absolute (a) and relative (b) CMAP amplitudes], **B**: strength-duration relationship, **C**: threshold electrotonus, **D**: current-threshold relationship, and **E**: recovery cycle. In Aa, arrows indicate stimulus currents for 50% maximal response.

Direct evidence for high affinity blockade of NaV1.6 channel subtype by huwentoxin-IV spider peptide, using multiscale functional approaches

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SUPPLEMENTARY TABLE 1

Table S1. Comparison of neuromuscular excitability parameters (means \pm S.D.) from mouse tail muscle recordings before toxin injections (control, n = 29 mice), ~30 min after injections of HwTx-IV (4.1-41.4 nmol/kg mouse, n = 6 mice) or TTX (5.3-17.8 nmol/kg mouse, n = 9 mice), and ~12 h after toxin injections (n = 12 mice).

Excitability parameter ²		Before toxin injection	~30 min after injection HwTx-IV	TTX	~12 h after toxin injection
A ¹	Peak response (mV)	5.31 \pm 0.57	0.92 \pm 0.31**	1.36 \pm 0.25**	5.04 \pm 0.56
A	Latency (ms)	3.89 \pm 0.06	4.09 \pm 0.26	4.09 \pm 0.12	3.91 \pm 0.04
A	Stimulus (mA) for 50% max response	0.35 \pm 0.05	0.46 \pm 0.05*	0.44 \pm 0.08*	0.36 \pm 0.06
A	Stimulus-response slope	3.11 \pm 0.18	3.42 \pm 0.17	2.96 \pm 0.17	3.21 \pm 0.16
B ¹	Strength-duration time constant (ms)	0.69 \pm 0.12	0.86 \pm 0.14	0.87 \pm 0.11	0.66 \pm 0.03
B	Rheobase (mA)	0.21 \pm 0.04	0.19 \pm 1.16	0.19 \pm 0.03	0.22 \pm 0.08
C ¹	TEd (10-20 ms, 40%)	59.53 \pm 1.27	54.84 \pm 1.58	59.19 \pm 1.84	56.07 \pm 2.07
C	TEd (peak, 40%)	59.00 \pm 1.17	63.62 \pm 1.43	60.52 \pm 1.96	56.28 \pm 1.92
C	TEd (peak, 20%)	35.81 \pm 0.75	38.65 \pm 1.69	36.14 \pm 1.03	33.57 \pm 1.53
C	TEd (40-60 ms, 40%)	47.92 \pm 1.14	51.90 \pm 1.09	50.17 \pm 2.03	43.29 \pm 2.29
C	TEd (90-100 ms, 40%)	39.52 \pm 1.27	41.49 \pm 1.25	42.20 \pm 1.67	36.56 \pm 2.41
C	Accommodation half-time (ms, 40%)	46.02 \pm 0.93	44.09 \pm 1.15	46.05 \pm 1.70	45.16 \pm 2.69
C	S2 accommodation	19.48 \pm 0.77	19.13 \pm 1.32	18.32 \pm 0.89	18.72 \pm 1.45
C	TEd (undershoot, 40%)	-15.34 \pm 1.01	-12.26 \pm 1.27	-17.67 \pm 1.35	-13.43 \pm 1.29
C	TEh (10-20 ms, -40%)	-93.31 \pm 1.12	-94.49 \pm 1.56	-91.87 \pm 2.14	-90.88 \pm 4.93
C	TEh (20-40 ms, -40%)	-120.1 \pm 1.9	-124.0 \pm 3.0	-119.8 \pm 3.4	-116.2 \pm 7.5
C	TEh (90-100 ms, -40%)	-152.7 \pm 5.2	-167.9 \pm 4.9	-156.7 \pm 5.6	-149.4 \pm 12.1
C	TEh (slope 101-140 ms, -40%)	2.62 \pm 0.17	2.74 \pm 0.11	2.93 \pm 0.20	2.60 \pm 0.30
C	TEh (peak, -70%)	-312.4 \pm 6.7	-321.8 \pm 11.0	-300.3 \pm 10.1	-297.8 \pm 22.8
C	S3 (-70%)	80.28 \pm 6.98	102.1 \pm 11.4	80.48 \pm 7.91	78.85 \pm 11.20
C	TEh (overshoot, -40%)	17.33 \pm 1.05	20.23 \pm 1.41	17.70 \pm 1.20	15.62 \pm 1.71
D ¹	Resting slope	0.89 \pm 0.09	0.86 \pm 0.12	0.89 \pm 0.11	0.96 \pm 0.26
D	Minimum slope	0.23 \pm 0.01	0.23 \pm 0.02	0.23 \pm 0.02	0.24 \pm 0.02
D	Hyperpolarization slope	0.49 \pm 0.03	0.39 \pm 0.05	0.40 \pm 0.06	0.48 \pm 0.05
E ¹	Refractoriness at 2 ms (%)	45.82 \pm 5.60	46.20 \pm 11.00	47.91 \pm 4.41	48.05 \pm 10.20
E	Refractoriness at 2.5 ms (%)	34.48 \pm 4.78	43.90 \pm 6.62	31.13 \pm 5.72	32.36 \pm 7.01
E	Relative refractory period (ms)	4.38 \pm 1.09	5.05 \pm 1.22	4.36 \pm 1.11	4.70 \pm 1.07
E	Superexcitability (%)	-19.00 \pm 3.91	-23.53 \pm 8.01	-17.94 \pm 3.74	-9.00 \pm 3.17
E	Superexcitability at 5 ms (%)	-0.49 \pm 3.26	3.98 \pm 2.84	-1.63 \pm 4.46	-2.40 \pm 1.75
E	Superexcitability at 7 ms (%)	-5.94 \pm 2.06	-1.78 \pm 1.69	-7.06 \pm 2.83	-7.01 \pm 1.76
E	Subexcitability (%)	5.60 \pm 1.09	3.29 \pm 0.85	6.61 \pm 1.46	2.93 \pm 0.64

¹ A: stimulus-response relationship, B: strength-duration relationship, C: threshold electrotonus, D: current-threshold relationship, and E: recovery cycle. ² TEd: threshold electrotonus from depolarizing currents and TEh: threshold electrotonus from hyperpolarizing currents. Differences (*versus* control), considered to be significant at $P \leq 0.05$ (*: $P \leq 0.032$, **: $P \leq 9.4 \times 10^{-8}$), are highlighted in grey.