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1 **Nav1.7 and pain: contribution of peripheral nerves**

2 Running title: Sensory phenotyping Nav1.7 knock-outs

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25

26 **Abbreviation list:**

27 Action potential (AP); Activity-dependent slowing (ADS); Afterhyperpolarization (AHP); A-
28 fiber compound action potential (A-CAP); C-fiber compound action potentials (C-CAP);
29 high-threshold mechanosensitive C-fiber (C-HTM); Congenital indifference to pain (CIP);
30 Calcitonin gene-related peptide (CGRP); low-threshold mechanosensitive C-fiber (C-LTM);
31 mechano-cold sensitive C-fiber (CMC); mechano-heat sensitive C-fiber (CMH); mechano-
32 heat-cold sensitive C-fiber (CMHC); Dorsal root ganglion (DRG); Knock – out mice (KO);
33 Receptive field (RF); Synthetic interstitial fluid (SIF); Tetrodotoxin (TTX); Tetrodotoxin
34 resistant (TTXr); Tetrodotoxin sensitive (TTXs); Transient receptor potential vanilloid 1
35 (TRPV1); Voltage-gated sodium channels (VGSC);.

36

37 **Key words: voltage-gated sodium channels, unmyelinated fibers, CGRP release,**
38 **compound action potential, plantar test, von Frey test**

39 INTRODUCTION

40 The TTX-sensitive sodium voltage-gated channel $Na_v1.7$, encoded by the *SCN9A* gene has
41 been in the center of latest research concerning pain mechanisms and the development of new
42 analgesics. Human gain-of-function mutations lead to erythromelalgia [35;60], small fiber
43 neuropathy [18;23;23;42] and paroxysmal extreme pain disorder [17;29;61], in all of which
44 pain perception is excessive. Increased channel expression and activity have also been
45 reported in peripheral neuropathic and diabetic rat models [19;28;33;49;52;59;62]. In contrast,
46 human loss-of-function mutations cause congenital indifference to pain (CIP) and anosmia
47 [1;13;22]. These symptoms are also present in mutant mice, both conditional and global
48 knock-outs, presenting with subnormal pain behavior in several pain models and behavioral
49 signs of anosmia [11;21;31;40;49;58].

50 In the olfactory system $Na_v1.7$ is expressed in the soma, axon and synaptic bouton of
51 olfactory sensory neurons [2;58]. Likewise, in the nociceptive system it is present in small
52 dorsal root ganglion neurons (DRG), their unmyelinated axons as well as cutaneous terminals
53 [7;41;46], and it is largely absent in the CNS [54], suggesting that initiation, conduction and
54 first synaptic transmission of action potentials might critically depend on $Na_v1.7$ and explain
55 the phenotype of knock-out animals and missense mutations in humans. However the fact that
56 the histamine-evoked axon reflex is unaltered in CIP patients [22], while itch perception is
57 absent [21], suggests that spike initiation and peripheral nociceptive conduction are at least
58 partly functional in spite of the absence of $Na_v1.7$. Likewise, it has recently been shown in a
59 CIP patient and $Na_v1.7$ conditional knock-out animals that naloxone can partially restore pain
60 [38]. This means that at least part of the peripheral input must reach the presynapse, but due to
61 synaptic inhibition induced by an upregulated opioidergic system in $Na_v1.7$ deficient
62 animals/humans is not transmitted further centrally. However, for the olfactory system of

63 $Na_v1.7$ -deficient animals transmission failure has been shown, action potentials properly
64 generated in sensory neurons do not initiate synaptic signaling [58].

65 These studies indicate that peripheral somatic nerves may still generate action potentials and
66 propagate, at least part of them, centrally in the absence of $Na_v1.7$. In the light of the
67 biophysical properties of $Na_v1.7$, its absence would be expected to have an influence on
68 initiated conduction of action potentials in peripheral nerves. Its relatively hyperpolarized
69 activation and inactivation voltage in comparison to $Na_v1.8$ [3;50] and its rapid
70 activation/inactivation kinetics [30] make it suited as an amplifier of subthreshold
71 depolarizations, setting the spike threshold. Furthermore, its slow repriming [24], its
72 prominently slow closed-state inactivation and recovery [14] and the ability to generate
73 resurgent currents [18] are indicative of determining discharge patterns and neural
74 accommodation.

75 So far, studies on pain pathways in $Na_v1.7$ global [21] or conditional knock-out mice
76 [36;37;40] focus on pain behavior or DRG discharge or currents. Here we compare generation
77 and conduction of action potentials and their accommodation in the peripheral sensory
78 endings and nerves of DRG-selective (Advillin/Cre) SCN9A knock-out and wild-type mice.
79 Apart from general physiological interest, for development of drugs targeting $Na_v1.7$ it is
80 essential to know whether a compound should engage the neuron before or behind the blood-
81 brain barrier.

82

83

84

85 MATERIALS AND METHODS

86 Chemicals and solutions

87 Synthetic interstitial fluid (SIF, [10]) solution used for single-fiber recordings, compound
88 action potential experiments and CGRP release measurements consisted (in mM) of: 107.8
89 NaCl, 26.2 NaCO₃, 9.64 Na-gluconate, 7.6 sucrose, 5.05 glucose, 3.48 KCl, 1.67 NaH₂PO₄,
90 1.53 CaCl₂ and 0.69 MgSO₄, continuously gassed with carbogen (95% oxygen and 5% carbon
91 dioxide) equilibrating the solution at pH 7.4. Addition of potassium for 60mM depolarizing
92 KCl solution was compensated by subtraction of an equimolar NaCl concentration.

93 Animals

94 The Advillin-Cre transgenic mice, were a generous gift from J. N. Wood [37]. Floxed
95 SCN9A^{loxP/loxP} mice were kindly supplied by T. Leinders-Zufall [58]. The Nav1.7 gene was
96 selectively deleted in sensory neurons expressing Advillin, by crossing Advillin-Cre with
97 SCN9A^{loxP/loxP} mice [37]. Genomic DNA from the tails of KO mice was genotyped using the
98 polymerase chain reaction (PCR). The following primers were used (Invitrogen) for Advillin-
99 Cre genotyping: primer1 (Adv forward) - CCCTGTTCACTGTGAGTAGG, primer2 (Adv
100 WT-reverse) - AGTATCTGGTAGGTGCTTCCAG and primer3 (Adv-Cre reverse) -
101 GCGATCCCTGAACATGTCCATC. Following a 3 min period at 96°C, DNA samples were
102 denaturated (96°C 30sec), annealed (63°C, 30sec) and extended (72°C, 1min) for 30 cycles
103 with a subsequent 10 min period at 72°C. The wildtype band from Primers 1+2 was 480 bp
104 long and mutant allele from Primers 1+3 was 180 bp long. The Primers used for loxP
105 genotyping: primer4 (SCN9A forward) - CAGAGATTTCTGCATTAGAATTTGTTTC,
106 Primer5 (SCN9A WT/floxed reverse) - AGTCTTTGTGGCACACGTTACCTC and Primer6
107 (SCN9A KO reverse) - GTTCCTCTCTTTGAATGCTGGGCA. Following a 2 min period at

108 94°C, DNA samples were amplified (94°C 30sec), annealed (60°C, 30sec) and extended
109 (72°C, 2min) for 34 cycles. Wildtype band from primers 4+5 was 317 bp long, loxP allele
110 from primers 4+5 was 461 bp long and a Nav1.7 KO allele was indicated by a 395 bp long
111 band from Primers 4+6. Both Advillin-Cre and SCN9A^{loxP/loxP} mice were continuously crossed
112 with C57BL6 to congenity so C57BL/6 mice were used as control animals [58]. Inbred
113 C57BL/6 and Nav1.7^{Adv} conditional KO mice of both sexes and ranging in weight between
114 20-25g were housed in group cages in a temperature-controlled environment with a 12h light-
115 dark cycle and were supplied with food and water *ad libitum*. Animals were killed in a rising
116 CO₂ atmosphere in accord with German and European laws.

117 **Transcriptional regulation**

118 Total RNA was extracted from homogenized dorsal root ganglia (10-20 ganglia per mouse, 3
119 mice from each genotype) with the RNeasy Mini kit and treated with DNaseI (QIAGEN,
120 Hilden, Germany). First-strand cDNA was synthesized from 150 ng oligo(dT)-primed RNA
121 with the RevertAid Reverse Transcriptase kit from Thermo Fisher Scientific. For quantitative
122 real-time RT-PCR (qRT-PCR) analysis, cDNA reactions were mixed with 2x Absolute QPCR
123 Mix, SYBR Green, Rox Mix (Thermo Fisher Scientific), the appropriate primers (from
124 Thermo Fisher Scientific) and filled with water to 20 µl. Nav1.7 Primer sequences are listed
125 above under “Animal” section, otherwise, the following Primers were used (all in 5' → 3'
126 direction): ACTB qRT-PCR forward – CGGTTCCGATGCCCTGAGGCTCTT, ACTB qRT-
127 PCR reverse – CGTCACACTTCATGATGGAATTGA. Expected product: 100 bp. SCN8A
128 qRT-PCR forward - CGTACTATTTGACGCAGAAAATT. SCN8A qRT-PCR reverse –
129 TCATGCTGAAGACTGAATGTATCA. Expected product: 153 bp. SCN9A qRT-PCR
130 forward – GCCTTGTTTCGGCTAATGAC, SCN9A qRT-PCR reverse –
131 TCCCAGAAATATCACCACGAC. Expected product: 111 bp. SCN10A qRT-PCR forward –

132 GTGTGCATGACCCGAACTGAT, SCN10A qRT-PCR reverse –
133 CAAAACCCTCTTGCCAGTATCT. Expected product: 101 bp. SCN11A qRT-PCR forward
134 – CCCCTGACCTTATAGCGAAGC, SCN11A qRT-PCR reverse –
135 CTCTTGGCGCTGAAGCGATA. Expected product: 112 bp. Primers were initially tested at
136 concentrations of 100, 300 and 900 nM and finally used at 300 nM. qPCRs including no-
137 template and reverse transcriptase-minus controls were performed in triplicates in an Applied
138 Biosystems 7300 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) with
139 15 minutes as initial stage at 95°C to activate the DNA polymerase, followed by 40 PCR
140 cycles of 95°C for 15 sec and 60°C for 1 minute. Dissociation curves were generated by
141 heating to 95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec. PCR products were
142 additionally separated on agarose gels and visualized by ethidium bromide staining.
143 Standardization was performed by quantification of the beta Actin gene as an endogenous
144 control and the $\Delta\Delta C_t$ method. A cut-off of above twofold increase/decrease was set as a
145 criterion for changes in gene expression. Statistical analysis was performed on at least three
146 independent experiments (i.e., mice) and results are shown as fold increase in Nav1.7 KO
147 mice vs. C57BL6 controls.

148 **Behavioral tests**

149 All animal experiments were approved by the responsible appropriate Animal Protection
150 Authority (Regierung von Unterfranken, Würzburg, Germany). Paw withdrawal latency
151 measurements were performed on both male and female 8-10 weeks old mice. All mice were
152 tested in a "blind" manner, with the person performing the measurements unaware of the
153 mouse genotype. After acclimatization to the testing environment (a few hours exposure to
154 the experimental environment in the two consecutive days prior to the experiment), mice were
155 subjected to repeated plantar mechanical and heat stimulation. Mechanical threshold of the

156 paw was determined using a dynamic plantar aesthesiometer (Ugo Basile, Gemonio, Italy). A
157 movable unit supplied with a von Frey filament (0.5mm diameter) applied linearly increasing
158 forces onto the plantar side of the hindpaw until withdrawal, at which point the threshold
159 force was recorded. Measurements were done on alternating feet (at least 6 times per mouse),
160 with at least 3 min pause between each stimulus, and results from each mouse were averaged.
161 For thermal stimulation, an infrared light beam was focused to the plantar side of the hindpaw
162 (Ugo Basile, Gemonio, Italy) through a grid floor on which the mice were allowed to walk
163 unrestricted. The beam was automatically switched off upon withdrawal of the paw, recording
164 the latency. Both left and right paw were measured alternately (at least 8 measurements per
165 mouse) with minimum 3 minutes between stimulations. Two radiant heat stimuli of different
166 intensity (7 and 9 in arbitrary units) were alternately applied and latencies for each intensity
167 were averaged. The total number of sensory stimuli per animal (mechanical + thermal) was
168 between 14 and 20.

169 **Single-fiber electrophysiology**

170 Single-fiber recordings from cutaneous C fibers of the saphenous nerve were obtained using
171 the isolated skin-nerve preparation as described previously [26;43].

172 Mechanosensitive receptive fields were mapped using a blunt glass rod. Once a distinct
173 receptive field was identified, electrostimulation was applied to the RF through a metal
174 microelectrode to determine the fiber's conduction velocity. Values of conduction velocity
175 were used for classification with a cut-off criterion of <1.4 m/s for unmyelinated (C) fibers
176 [6]. Nav1.7 is primarily expressed in small DRGs and unmyelinated nerve fibers [7;46] thus,
177 electrophysiological recordings in this study were focused solely on unmyelinated C fibers.
178 Lack of initial spontaneous activity was a prerequisite for further testing of all units. A

179 marking technique was applied, in which latency shifts are provoked through simultaneous
180 application of mechanical and electrical stimuli to the RF [48], ensuring recording from a
181 distinct single-fiber. To assess sensory properties, the mechanical threshold of fibers was
182 characterized using calibrated von Frey (polyamide) bristles ranging in force from 1-128 mN
183 in a geometric scale and equipped with varnished tips (\varnothing 0.8mm). Subsequent to
184 characterisation, a metal ring (9 mm diameter) was placed encircling the RF. Vaseline was
185 applied to the base of the ring to improve fluid isolation. The fluid volume within the ring was
186 replaced by ice-cold buffer for noxious cold stimulation, or heated by a thermode coupled to a
187 custom-made Peltier device for noxious heat stimulation (20 s ramp of 32 ° - 46 °C). Each
188 stimulus was followed by a resting period of several minutes prior to onset of the following
189 stimulus to minimize the risk of sensitizing effects between modalities. Fibers were
190 considered cold/heat responsive if they produced at least 2 spikes concurrent to stimulation
191 onset. Heat threshold was defined as the temperature at which the second spike of the heat
192 response occurred.

193 The electrical excitability of the cutaneous nerve endings was determined as the voltage
194 threshold for eliciting an action potential. Rectangular constant voltage stimuli of variable
195 width (in ms: 0.02, 0.2, 2, 20, 200) were applied to the most mechanosensitive spot within the
196 receptive field. Activity-dependent slowing (ADS) of conduction velocity was measured
197 during an electrical stimulation protocol consisting of a 3 min pause, 6 min 0.25 Hz, 3 min 2
198 Hz, 6 min 0.25 Hz [20]. Changes in conduction velocity are expressed as percentage of initial
199 values (at stimulation onset) of the individual nerve. Maximal slowing is calculated for the
200 last 30 sec of the 2 Hz stimulation. In addition, a recovery cycle protocol at 0.2ms and
201 supramaximal strength was performed, [9;57]. In a continuous stimulation frequency of 0.5
202 Hz single conditioning pre-pulses at different inter-stimulus intervals (6-500 ms) were

203 interposed and the latency shift of the conditioned action potential as compared to its
204 conditioning predecessor was analyzed. Finally, some of the fibers were electrically
205 challenged at the RF with ongoing paired electrical pulses (20 ms intrastimulus intervals) at 2
206 Hz [20;56] and 2-3 fold individual threshold voltage (increased if necessary to compensate for
207 a possible drift of electrical threshold). This provides the time required to provoke conduction
208 block as an index of conduction reliability. Conduction block was defined as the condition
209 when the paired pulses ceased to yield two discernible spikes.

210 **Compound action potential recordings**

211 Compound action potential (CAP) signals were recorded extracellularly from isolated
212 segments of mouse saphenous nerve. The saphenous nerves from C57BL/6 and $Na_v1.7^{Adv}$
213 knock-out mice were dissected bilaterally from their point of leaving the inguinal region to
214 approximately 5mm below the knee. Nerves were desheathed to remove the epi- and
215 perineurium and placed in a recording chamber between two suction electrodes. The bath was
216 perfused continuously with SIF, bubbled continuously with carbogen (95% O_2 : 5% CO_2) to a
217 pH of 7.4. Experiments were performed in room temperature as at this temperature the
218 superimposed action potential was still above detection level in all nerves. For electrical
219 stimulation and recording the cut ends of the nerve were pulled through a silicon membrane,
220 creating an optimal seal of the recording and stimulating neuronal sites. The chamber is a
221 modification of the previously published one [12]. Silver wire electrode served as the cathode
222 and anode for stimulation for one suction electrode and for a differential recording at the
223 other. CAP responses were evoked using constant current stimulation (A395, WPI, Sarasota,
224 USA) of fixed duration (1 ms) For the determination of amplitude and conduction velocity
225 (i.e conduction latency over the fixed recording distance of 5 mm between electrodes) the C-
226 fibre CAP response to supra-maximal electrical stimulation was assessed. To elucidate CAP

227 changes in response to a repetitive stimulation challenge, a stimulus protocol was used
228 comprising 3 min pause followed by 6 min at 0.25 Hz, 3 min at 2.5 Hz, 6 min at 0.25 Hz (1/4
229 s). Changes in conduction latency and CAP amplitude during this protocol are expressed as
230 percentage change of their initial value at stimulation onset, i.e. relative to the first value after
231 the 3minute pause.

232 **CGRP release**

233 Flaps of the hairy skin from the lower leg and foot were excised and wrapped around acrylic
234 glass rods (6mm diameter) with the corium side exposed, as previously described [47].
235 Samples were placed in carbogen gassed SIF (equilibrating the solution at pH 7.4) and
236 positioned in a shaking bath set to 32°C for a washout period of 30min. Skin flaps were then
237 consecutively passed through a set of 4 glass tubes containing 800µl SIF. Each incubation
238 step lasted 5min. The first 2 incubation steps were to determine basal CGRP release at 32°C.
239 The third incubation step assessed stimulus-induced CGRP release and the reaction tubes
240 contained either SIF at 47°C or SIF with 60mM KCl. The fourth incubation step assessed
241 recovery of the response in SIF solution at 32°C. CGRP levels were determined using
242 commercial enzyme immunoassays (EIAs; Bertin Pharma, Montigny, France), as previously
243 described [5]. Samples were photometrically analyzed using a microplate reader (Opsys
244 MRTM, Dynex Technologies, Chantilly, Virginia, USA).

245 **Statistics**

246 Statistical analysis was performed using the Statistica software package 7.0 (Statsoft). For
247 multiple groups comparison one-way analysis of variance (one-way ANOVA) was used.
248 Other statistical tests used are denoted in the text. $P < 0.05$ was considered statistically
249 significant (depicted as * in the figs). All data is presented as mean \pm SEM.

250 **RESULTS**

251 **Behavioural tests in naive animals**

252 Behavioral tests in animals represent the integrative performance of both central and
253 peripheral nervous systems. We tested the mechanical and heat sensitivity of Nav1.7^{Adv}
254 conditional KO vs. congenic control mice. From their general appearance, control animals
255 could not be differentiated from KOs, which were as heavy, big, groomed and vigilant as
256 controls. By a blinded examiner, two heat stimulus intensities were tested in each group, both
257 revealing a significantly longer withdrawal latency in Nav1.7^{Adv} KOs (Fig. 1A): 27.4 ± 3.3
258 sec for the lower stimulus intensity (7 arbitrary units) and 25.5 ± 3 sec for the higher intensity
259 (9 arbitrary units), versus 14 ± 2.6 sec and 12.6 ± 2.8 sec, respectively, in controls ($p < 0.05$ U-
260 Test). Though less substantial, the mechanical threshold (dynamic von Frey) was also
261 significantly elevated in KOs (Fig. 1B): 4.2 ± 0.7 mN versus 3.1 ± 0.6 mN in controls ($p < 0.05$
262 U-Test).

263 **Recordings from unmyelinated cutaneous single-fibers**

264 The lack of Nav1.7 in peripheral sensory neurons resulted in reduced behavioural noxious heat
265 and mechanical responsiveness of mice. We therefore tested whether this deficiency would be
266 recapitulated in the properties of the cutaneous nerve endings. 60 mechanosensitive cutaneous
267 C-units from controls and 30 from Nav1.7^{Adv} KOs were recorded. The fiber population from
268 controls comprised (Fig. 2A): 45 mechano-heat sensitive C-fibers (CMH; 75%), 10 C high
269 threshold mechanosensitive fibers (C-HTM; 17%), 2 low-threshold mechanosensitive C-fibers
270 (C-LTM; 3%), 2 mechano-cold sensitive C-fibers (CMC; 3%) and 1 mechano-heat-cold C-
271 fiber (CMHC; 2%). The fiber population in Nav1.7^{Adv} KO was significantly different from
272 that of controls, with mechano-heat sensitive fibers no longer being the most abundant (χ^2 (df

273 = 4, n= 90) = 15, $p < 0.001$): 10 CMH fibers (33%), 13 C-HTM fibers (43%), 4 CMC (13%), 2
274 C-LTM (7%) and 1 CMHC (3%). The sensory capacities (mechanical and thermal) of the
275 fiber populations from both genotypes were compared. The mechanosensitivity of the KO
276 units did not significantly differ from that of controls (Fig. 2B-C). The 10 $\text{Nav}1.7^{\text{Adv}}$ KO C-
277 fibers that were heat sensitive surprisingly seemed more responsive to heat than the control
278 units (with higher discharge rate during stimulation, though not significant, Fig. 2D) and a
279 lower mean heat threshold in comparison to controls (37.9 ± 1.5 °C and 40.3 ± 0.4 °C,
280 respectively, Fig. 2E, $p=0.059$, U-Test).

281 We next examined the conductive properties of the single units. The mean conduction
282 velocity of KO fibers was significantly lower than that of controls by about 20%, while the
283 variance was about the same (Fig 3A-B, 0.4 ± 0.04 m/s vs. 0.5 ± 0.02 m/s, respectively
284 ($p < 0.05$ U-Test). The single-fiber recordings from $\text{Nav}1.7$ KO mice have not shown reduced
285 sensitivities to the diverse modalities of mechanical and heat stimulation. A possible
286 explanation for that would be that the voltage threshold for triggering propagated action
287 potentials in the skin nerve endings is about the same in KOs and WTs. We therefore assessed
288 the electrical excitability, placing an electrode (cathode) in the receptive field at the spot of
289 highest mechanosensitivity. In addition to varying the stimulus voltage, we also varied the
290 stimulus duration in order to gain information about a potential kinetic difference between KO
291 and WT nerve terminals. However, the resulting strength-duration curves did not significantly
292 differ between the genotypes (Fig. 3C, left graph, multiple T-tests with Bonferroni
293 correction). Also the chronaxy did not appear different between KO and WT. This became
294 evident when the parabolic curve was linearized by plotting the product of threshold voltage
295 and stimulus duration (an analogue to threshold charge transfer) over stimulus duration in a
296 linear coordinate system (Fig. 3C right graph). In this representation the chronaxy

297 corresponds to the abscissa intercept, which is obviously the same in both genotypes, whereas
298 the slightly different slopes of the (regression) lines correspond to the non-significant
299 difference of the rheobases. This means that the kinetic requirements (utilization times) of the
300 electrical stimulus to trigger an action potential are not different between Nav1.7^{-/-} and WT.
301 In other words, Nav1.7 is not required as an action potential generator in those C-fibers that
302 retained mechanosensitivity and electrical excitability in the KOs.

303 The recovery cycle experiment (Fig. 3D) with electrical double pulses at varying inter-
304 stimulus intervals (ISI) studies the impact of a preceding action potential on the conduction
305 velocity (latency) of the subsequent, second, spike. Apart from the prolonged latency (slower
306 conduction velocity) in KOs vs. WTs, visible at longer ISI (> 100 ms), the exponential
307 increase of the latency with shorter ISIs (< 100 ms) in the KO fibers is striking and obviously
308 different from the moderate increase in WT fibers. These changes are attributed to long
309 lasting afterpotentials, most probably of hyperpolarizing direction. Thus, Nav1.7 seems to
310 stabilize the conduction velocity at shorter spikes intervals (higher discharge rates).

311 This role of Nav1.7 in stabilizing conduction velocity lead us to examine the activity-
312 dependent slowing of conduction velocity (ADS) upon prolonged low frequency (e.g. 2 Hz)
313 stimulation. This phenomenon is due to progressive slow inactivation of the voltage-gated
314 sodium channels and to accumulation of sodium ions in the thin nerve fibers [15;53]. During a
315 control period of 6 min at 0.25 Hz the fibers became slightly slower in conduction, showing
316 no difference between both genotypes (Fig 4A). During the more frequent electrical
317 stimulation at 2 Hz for 3 min, the ADS became progressively prominent in the WTs, but the
318 KO fibers soon lagged behind and finally reached clearly less maximal ADS than the WT
319 units (Fig. 4B: $35.5 \pm 6.2\%$ vs. $57.4 \pm 4.7\%$, respectively, $p < 0.05$, U-Test). During the
320 subsequent recovery period of 6 min at 0.25 Hz both latencies returned towards their original

321 values. Progressive ADS of single units often leads to conduction failures, in which condition
322 fibers do not reliably respond to each electrical stimulus with a propagated action potential.
323 Our stimulation test was a 4 min period of 2 Hz double-pulse (ISI 20 ms) stimulation. In
324 accord with the finding of less ADS, the KO fibers were much less prone to block in
325 comparison to controls, more reliably responding to the second of the paired stimuli (Fig. 4C,
326 block of 6/13 units as opposed to 12/13, respectively).

327 In conclusion and comparison to WT fibers, the mechanosensitive cutaneous C-fibers that we
328 could identify in $Na_v1.7^{Adv}$ KO mice, exhibited (1) a significantly lower prevalence of
329 responsiveness to moderate noxious heat, although (2) the rarer heat responses seemed to
330 show exceptionally high mean discharge rates and low heat thresholds; (3) mechanosensitivity
331 was about normal. In biophysical respects, (4) conduction velocity was 20% slower and (5)
332 became progressively slower in response to the second of two electrical stimuli applied at
333 short interval (< 100 ms); (6) electrical excitability, rheobase and chronaxy, were about
334 normal. Upon prolonged electrical stimulation at 2 Hz, (7) activity-dependent slowing of
335 nerve fiber conduction was markedly less expressed and (8) more rarely led to conduction
336 failure of the KO single-fibers. Above all, it seems that a subpopulation of moderately heat
337 responsive polymodal nociceptors is, at least functionally missing in $Na_v1.7^{Adv}$ mice, while
338 the retained and detectable C-fibers in these mice exhibit anomalous biophysical properties
339 that do not indicate any susceptibility to failure of action potential generation or conduction.

340 **Stimulus-induced cutaneous CGRP release**

341 The single-fiber recordings suggested the absence of a subpopulation of heat sensitive
342 nociceptors in $Na_v1.7^{Adv}$ KO mice. A way to test the overall heat responsiveness of a skin flap
343 is to measure stimulated CGRP release which can serve as an index for the activation of, at
344 least, the peptidergic neurons [26]. However, hindpaw skin flaps of $Na_v1.7^{Adv}$ KO and control

345 mice revealed no significant difference in CGRP release evoked by heating to 45°C or, for
346 control, by depolarisation with 60mM KCl (Fig. 5). This CGRP release depends on calcium
347 influx through heat-activated ion channels or voltage-gated calcium channels, respectively,
348 but not on action potential generating sodium channels [51]. Thus, the results exclude the
349 absence of a peptidergic subpopulation of fibers, e.g. for possible developmental reasons in
350 the transgenic mice, but they do not exclude the functional inability of these fibers to generate
351 propagated action potentials.

352 **Compound action potential recordings**

353 The single-fiber recordings from the saphenous innervation territory suggested both the
354 functional absence of a whole subpopulation of conducting C-fibers and altered conduction
355 properties of the retained C-fibers as a result of the conditional deletion of $Na_v1.7$ in the
356 DRGs. Both consequences should show up in whole saphenous nerve recordings of the
357 compound action potentials. Indeed, the A-fiber CAP amplitudes were about the same in KOs
358 and WTs, but the C-CAPs of the KO mice were by two thirds smaller and half as fast in
359 conduction velocity than in the WTs (Fig. 6A-C). These deficits can hardly be due to
360 dispersion (in time) of the electrically evoked action potentials, as the single-fiber spikes
361 showed about the same amplitudes and even slightly less scattering of the reduced conduction
362 velocities in KOs as compared to WTs (see above). Also the finding of less ADS in KOs than
363 WTs was fully recapitulated in the C-CAP recordings (Fig. 6D).

364 **Transcriptional regulation**

365 It has previously been shown that in $Na_v1.8$ KO mice there is transcriptional upregulation of
366 $Na_v1.7$ mRNA [4], also of relevance for neuronal excitability in whole animals [34;63]. To
367 make sure our results do not stem from similar transcriptional upregulation we have screened

368 KOs for expression of specific sodium channels. Quantitative PCR results from DRGs
369 demonstrate that the conditional KOs had no significant change in gene expression of $Na_v1.6$,
370 $Na_v1.8$ and $Na_v1.9$ in comparison to control mice (Fig 7).

371

372

373 **DISCUSSION**

374 Na_V 1.7 human mutations cause either extremely painful diseases or indifference to pain
375 without further neurological deficits except for anosmia. Several studies have used knock-out
376 models of Na_V 1.7 to elucidate the phenotype related to the gene deletion with behavioural
377 testings or recordings from second order lamina V neurons in the spinal dorsal horn of
378 primary neurons [21;37;40]. In contrast, we focused on the hypothesis of a subthreshold
379 amplifying function of $Nav1.7$. We found no difference in the mechanical thresholds between
380 KOs and WTs and heat sensitive fibers the KOs did not present reduced, but rather
381 insignificantly enhanced heat responsiveness. This was most likely due to the fact that more
382 than half of the "normal" polymodal nociceptors with moderate heat responses (< 25 spikes in
383 a 20 s ramp from 32°-46°C) were missing in the KO skin. In their place the fraction of high-
384 threshold mechanosensitive and mechano-cold sensitive C-fibers was relatively enlarged from
385 20% in WT to 56% in the KOs.

386 A central question is whether these typical polymodal C-fibers are physically absent in
387 Na_V 1.7Adv KOs, or just unable to generate/ propagate action potentials along their axons? At
388 least one essential function; neuropeptide (CGRP) release from cutaneous sensory axons and
389 terminals was largely unaffected in the KOs. This important function requires the presence of
390 peptidergic nerve fibers connected by axonal transport to their DRG cell bodies, but it does
391 not require action potential discharge, just depolarization and calcium influx are sufficient
392 [51]. The axon reflex erythema upon histamine injection is reported to be functional in human
393 CIP mutants [22]. This suggests that action potentials are generated and (antidromically)
394 conducted through wide branching arborisation of the CGRP-expressing nerve fibers. It does
395 however not mean that these action potentials are propagated up the peripheral nerve.

396 Our previous studies with pharmacological elimination of $\text{Na}_v 1.7$ by TTX indicate that
397 actually almost all cutaneous C-fibers are more or less affected by the toxin, at least at higher
398 temperatures [63]. This raises the question whether the subpopulation of single-fibers
399 compensating the lack of $\text{Na}_v 1.7$ by other channels such as $\text{Na}_v 1.8$ or 1.9 in our present
400 study show biophysical symptoms resulting from the $\text{Na}_v 1.7$ deletion. An interplay of the two
401 major players $\text{Na}_v 1.7$ and $\text{Na}_v 1.8$ has been made responsible for electrogenesis in DRG
402 neurons [44]. While $\text{Na}_v 1.7$ opens in the subthreshold range and amplifies slow small
403 depolarisations (often together with $\text{Na}_v 1.9$) $\text{Na}_v 1.8$ takes over at more depolarised
404 membrane potentials to finally carry most the APs sodium influx ([25], for review see [55]).
405 Rapid repriming and relative resistance to inactivation make $\text{Na}_v 1.8$ also responsible for
406 conduction safety [27]. However this interplay depends on cell type. Rush and colleagues
407 have shown, that an erythralgia gain of function mutation of $\text{Na}_v 1.7$ (L858H) may result in
408 hyperexcitability of DRG neurons but hypoexcitability of sympathetic neurons [45]. Likewise
409 neurons – or even sections of one neuron (soma, axon, terminal) - with different expression
410 patterns or availability patterns (i.e. inactivity patterns at various resting membrane potentials)
411 of sodium channels ($\text{Na}_v 1.8$ in the above example) might modulate this interplay.

412 Changes of electrogenesis in the axon is expected to have an influence on conduction
413 velocity. In modelling studies conduction velocity (of myelinated fibres) has been predicted to
414 be mainly influenced by sodium channel kinetics i.e. composition of kinetically different
415 subtypes is more important than overall channel density [39]. Indeed, we found conduction
416 velocity of $\text{Na}_v 1.7$ deficient axons to be significantly lower by 20% than in WT fibers. Using
417 the same methods, we could not see altered conduction velocity in $\text{Na}_v 1.8$ [27] or $\text{Na}_v 1.9$ [26]
418 knock out animals. However unmyelinated axons lacking $\text{Na}_v 1.6$ [8] also slow down
419 conduction of compound action potentials by ~30%. A lack of the persistent current of $\text{Na}_v 1.6$

420 might have hyperpolarised these axons and thereby slowed down conduction. Also our results
421 suggest hyperpolarisation of $\text{Na}_v1.7$ deficient terminals. The recovery cycle experiments
422 revealed a pronounced sub-normality at double-pulse intervals shorter than 100 ms in the
423 KOs, much less in the WTs. This is reminiscent of the exceptionally long after-
424 hyperpolarizations, up to 100 ms, that DRG neurons with nociceptive C-fibers show [32].
425 Indeed, the conduction velocity of the units was significantly lower by 20% than in WT
426 fibers. The recovery cycle experiments revealed a pronounced sub-normality at double-pulse
427 intervals shorter than 100 ms in the KOs, much less in the WTs. This is reminiscent of the
428 exceptionally long after-hyperpolarizations, up to 100 ms, that DRG neurons with nociceptive
429 C-fibers show [32]. If this finding also applies to nerve endings, $\text{Na}_v1.7$ could rapidly recover
430 from fast voltage-dependent inactivation during the AHP and quickly respond to the
431 subsequent electrical stimulus, due to its relatively hyperpolarized activation range. If,
432 however, $\text{Na}_v1.8$ has to initiate the action potential, as in the KOs, it will take longer to reach
433 its more depolarized activation voltage range starting from a still hyperpolarized membrane
434 potential and, accordingly, the conduction velocity will drop. The underlying difference in
435 voltage-dependent activation between $\text{Na}_v1.7$ and $\text{Na}_v1.8$ is well established in DRG neurons
436 [16]. However, it does not seem to apply to the unmyelinated nerve endings in the skin, as the
437 parameters of electrical excitability, rheobase and chronaxy, were not significantly different
438 between $\text{Na}_v1.7\text{Adv}$ KOs and WTs. This should not be due to an insufficient power of
439 resolution of the extracellular electrostimulation technique, because the expectable reduction
440 of excitability in global $\text{Na}_v1.9$ KOs could recently be demonstrated using the same threshold
441 tracking technique [26]. Thus, the reason for the discrepancy between DRG cell bodies and
442 their cutaneous terminals remains unclear but may relate to the fundamental difference in
443 membrane surface – to – volume ratio.

444 The experiments on activity-dependent slowing of the conduction velocity refer to a different
445 time scale than the recovery cycle trials, the interstimulus interval was 500 ms and the
446 challenge of the C-fibers by continuous electrical stimulation lasted for 3 min. During this
447 period the ADS in WTs finally reached a plateau which was significantly lower in the KOs.
448 Two parallel mechanisms seem to account for the ADS phenomenon, an accumulation of
449 slow voltage-dependent inactivation of the sodium channels and an accumulation of sodium
450 ions inside the thin axons which reduces their inward driving force [15;53]. We tend to
451 assume that the absence of the $Na_v1.7$ -carried sodium current, reducing the accumulation, is a
452 reason for less ADS in the KOs. Both mechanisms can obviously lead to conduction failures,
453 in particular at branch points. This affected every single one of the WT fibers but only half of
454 the KO units during a 4 min challenge with 2 Hz double pulses. Again, we assume that less
455 sodium accumulation is responsible for this increased safety factor of conduction in the KOs.
456 We found many functional nociceptive C-fibers in the saphenous of $Na_v1.7^{Adv}$ KOs but also
457 an indication that one heat sensitive subpopulation of units was reduced, either physically
458 absent or unable to conduct action potentials. Our compound action potential recordings
459 cannot decide between these alternatives, but they suggest that a subpopulation of C-fibers is
460 not conducting at normal peripheral skin temperature (32°C). A principal problem with CAPs
461 is that their amplitude can shrink either by enhanced dispersion of the individual fiber
462 conduction velocities or by reduced size or number of the superimposed action potentials.
463 However, the single-fiber recordings did not reveal a greater scattering of the spike latencies
464 in the KOs and the spike amplitudes appeared normal with respect to the signal-to-noise ratio.
465 The conduction velocities were slower than in WTs, but this was also reflected by the C-CAP
466 which was equally slower in conduction in the KOs. Thus, most probably, it is the number of

467 superimposed C-fiber APs that is reduced in the KOs, in contrast to the A-fibers, for which A-
468 CAPs were not different between KO and WTs.

469 In synopsis of our results, we were surprised to find plenty of largely normal C-fiber
470 nociceptors in a transgenic mouse model of the human congenital indifference to pain (CIP)
471 syndrome. There is still a long distance to cover up from the peripheral saphenous nerve to the
472 spinal dorsal horn synapses, and the T-junction in the DRG as well as many branch points in
473 the dorsal horn have to be overcome by the action potentials. It seems that a subpopulation of
474 peripheral C-fibers is able to generate but not to properly propagate action potentials as in
475 primary olfactory neurons of conditional Nav1.7 KO mice that are able to generate action
476 potentials but not to initiate synaptic signalling in the olfactory bulb [58]. Even though
477 upregulation of opioidergic inhibition has recently been made responsible for reduced
478 nociceptive input in CIP [38], this cannot be expected to be the only reason as it cannot
479 explain anosmia. The subpopulation of peripheral C-fibres shown in this study to depend on
480 Nav1.7 for action potential propagation would also be expected to reduce nociceptive input by
481 drugs targeting Nav1.7 without crossing the blood-brain barrier.

482

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489 Conflict of interest: The authors declare no conflict of interest.

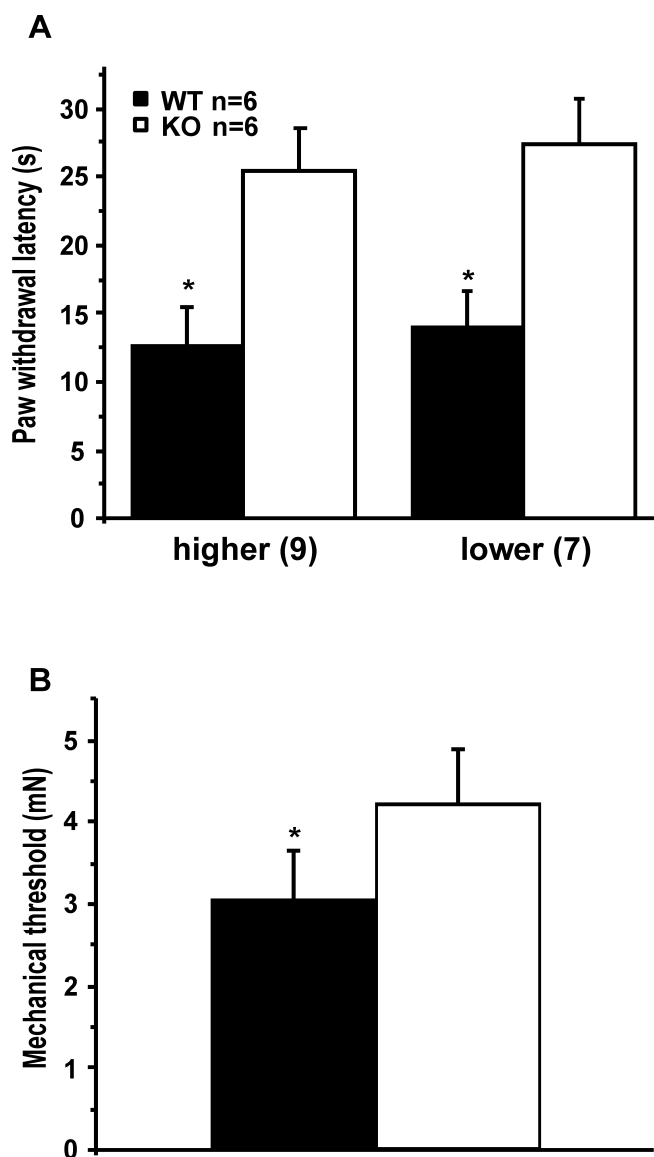
490 **FIGURES/LEGENDS**

491 **Fig. 1: Behavioural tests in conditional $\text{Na}_v1.7^{\text{Adv}}$ KO vs. congenic WT control mice. A.**

492 Blind testing of radiant heat-induced hindpaw withdrawal (Hargreaves' test) using two

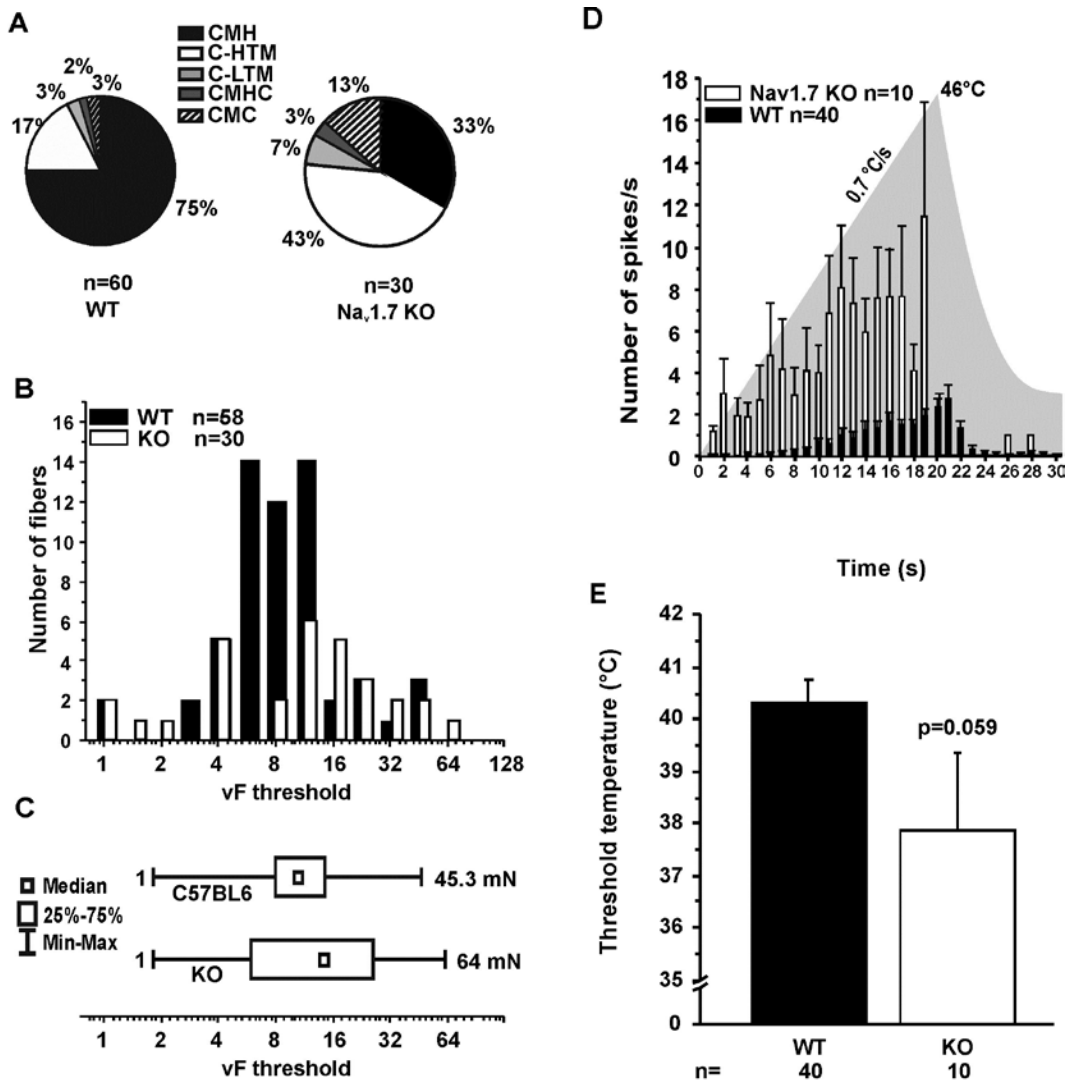
493 stimulus intensities (7 and 9, arbitrary units). B. Dynamic von Frey testing of mechanical

494 hindpaw withdrawal thresholds. Asterisks indicate $p < 0.05$ using U-test.



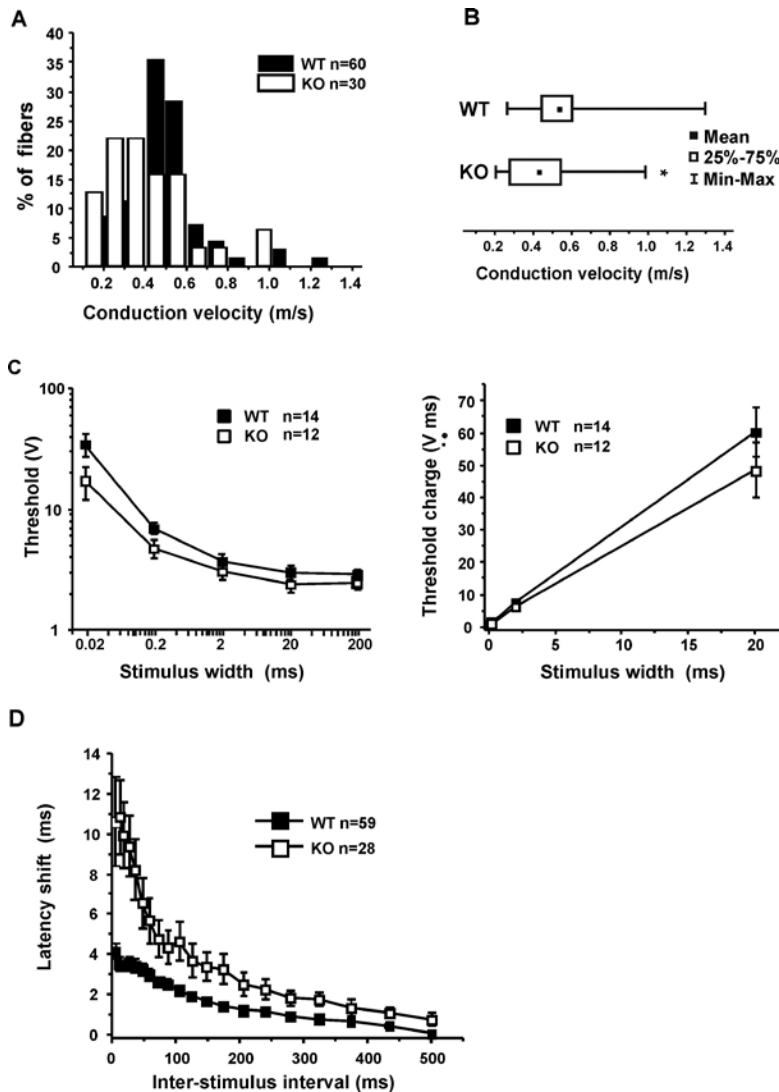
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496 **Fig. 2: Cutaneous C-fiber populations and sensory properties in $Na_v1.7^{Adv}$ KOs vs.**
 497 **WTs. A.** Sensory categorization of mechanosensitive unmyelinated single-fibers *in vitro*
 498 reveals differential prevalence between the genotypes, in particular with respect to heat
 499 sensitivity (χ^2 (df = 4, n= 90) = 15, p< 0.001). **B.** Distribution of dynamic von Frey thresholds
 500 in KOs and WTs over a geometric force scale. **C.** Boxplot of the mechanical thresholds. **D.**
 501 Averaged discharge rates per second in response to a 20 s radiant heat ramp (grey
 502 background); large variability in KO fibers due to small sample size. **E.** Mean heat thresholds
 503 of the units (temperature at second spike, U-test).



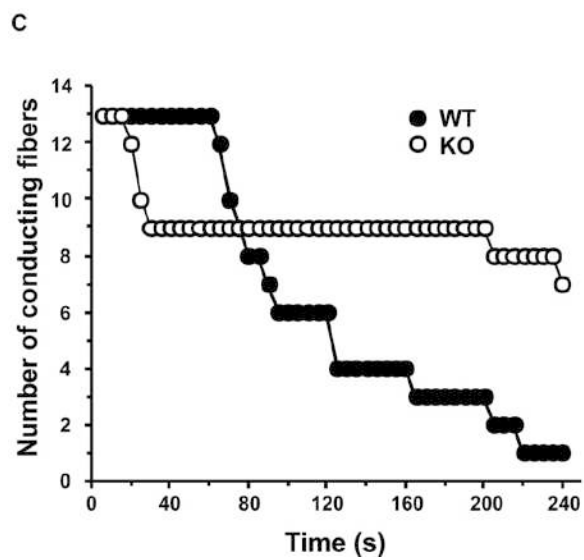
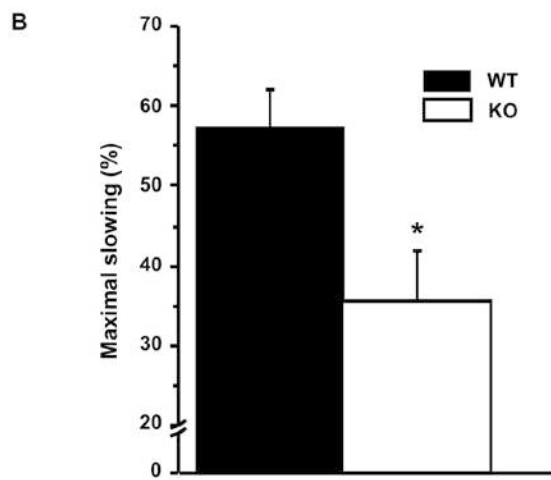
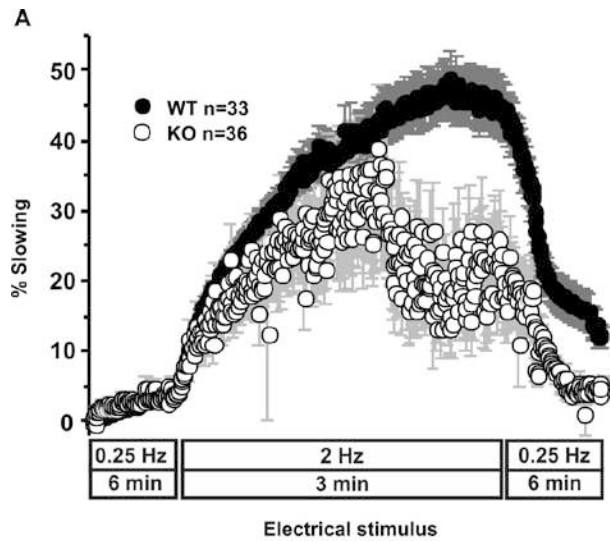
504

505 **Fig. 3: Conductive properties of C-fibers in KOs and WT.** **A.** Distribution of conduction
506 velocities over the distance from cutaneous receptive field to recording electrode. **B.** Modified
507 boxplot showing mean instead of median; asterisk indicates $p < 0.05$, U-test. **C.** Strength-
508 duration curves determined by threshold tracking with electrode in the receptive field (left
509 graph). Right graph: The parabolic curves in the left graph are linearized by calculating an
510 analog to threshold charge transfer ($V \cdot ms$), showing the slopes (i.e. rheobases) of the
511 regression lines and identical abscissa intercepts (i.e. chronaxies). **D.** Electrical double pulses
512 at varying ISI reveal differential retardation of the second spike, in particular at short intervals
513 ($p < 0.05$, multiple T-tests with Bonferroni correction).



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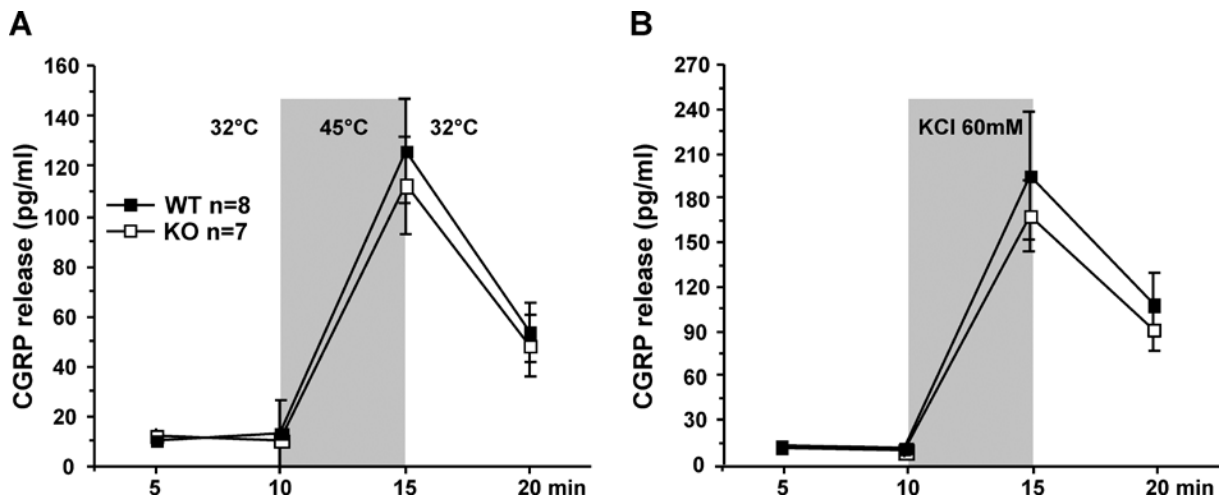
515 **Fig. 4: Activity-dependent slowing and propagation safety of C-fibers in KOs and WTs.**
516 **A.** Differential ADS development over time in percent of initial latency. **B.** Maximal ADS,
517 mean (\pm SEM) during the last 30 s of the 2 Hz stimulation period, the asterisk indicates
518 $p < 0.05$, U-test. **C.** Conduction failures during 4 minutes of electrical double pulse (20 ms ISI)
519 stimulation at 2 Hz. The decreasing number of fibers reliably conducting over time is
520 depicted.



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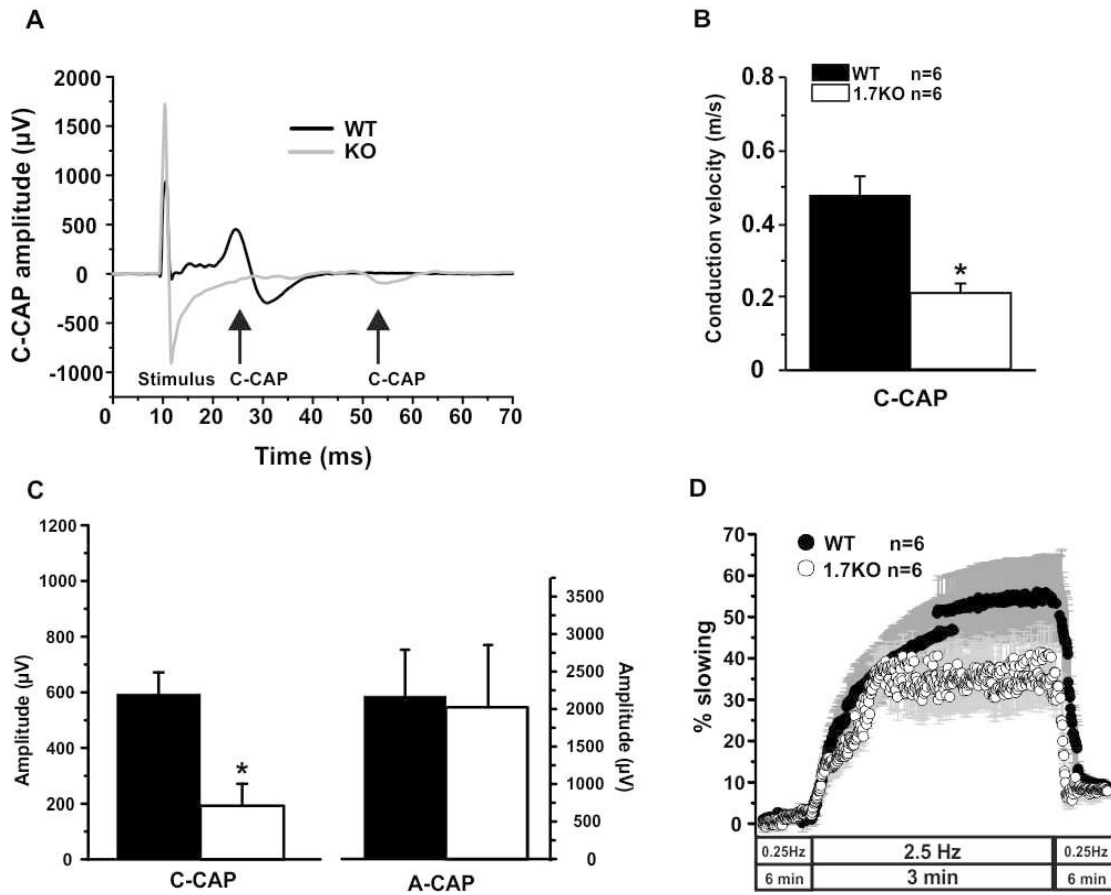
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523 **Fig. 5: Stimulated CGRP release from isolated skin. A.** Response to 45°C heat stimulation.
 524 **B.** Response to unspecific depolarisation with 60 mM external KCl.



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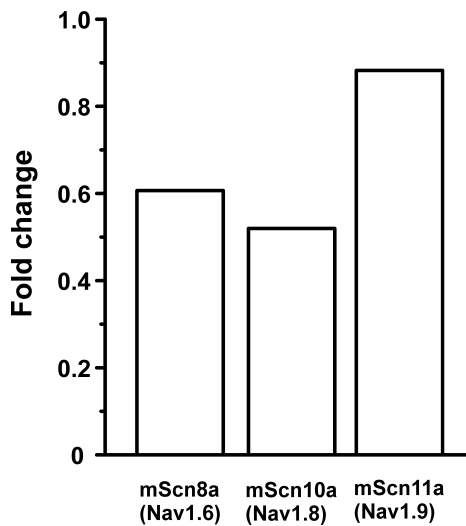
529 **Fig. 6: Compound action potentials of isolated saphenous nerves of KOs and WTs. A.**
 530 Single nerve examples showing fused electrical artefact and A-fiber CAPs as well as C-CAPs
 531 of differential latency and amplitude at 32°C. **B.** Conduction velocity of C-Caps, the asterisk
 532 indicates $p < 0.05$, U-test. **C.** Peak-to-peak amplitudes of C- and A-CAPs, the asterisk
 533 indicates $p < 0.05$, U-test. **D.** Development of ADS of the C-CAPs.



534

535 **Fig. 7: Gene expression in DRGs from Nav_{1.7} KO mice. Results are shown as fold change**

536 of gene expression in KOs vs. control mice.



537

538

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