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**Na\textsubscript{v}1.7 and pain: contribution of peripheral nerves**

Running title: Sensory phenotyping Na\textsubscript{v}1.7 knock-outs

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Abbreviation list:

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

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

The TTX-sensitive sodium voltage-gated channel Na\textsubscript{v}1.7, encoded by the SCN9A gene has been in the center of latest research concerning pain mechanisms and the development of new analgesics. Human gain-of-function mutations lead to erythromelalgia [35;60], small fiber neuropathy [18;23;23;42] and paroxysmal extreme pain disorder [17;29;61], in all of which pain perception is excessive. Increased channel expression and activity have also been reported in peripheral neuropathic and diabetic rat models [19;28;33;49;52;59;62]. In contrast, human loss-of-function mutations cause congenital indifference to pain (CIP) and anosmia [1;13;22]. These symptoms are also present in mutant mice, both conditional and global knock-outs, presenting with subnormal pain behavior in several pain models and behavioral signs of anosmia [11;21;31;40;49;58].

In the olfactory system Na\textsubscript{v}1.7 is expressed in the soma, axon and synaptic bouton of olfactory sensory neurons [2;58]. Likewise, in the nociceptive system it is present in small dorsal root ganglion neurons (DRG), their unmyelinated axons as well as cutaneous terminals [7;41;46], and it is largely absent in the CNS [54], suggesting that initiation, conduction and first synaptic transmission of action potentials might critically depend on Na\textsubscript{v}1.7 and explain the phenotype of knock-out animals and missense mutations in humans. However the fact that the histamine-evoked axon reflex is unaltered in CIP patients [22], while itch perception is absent [21], suggests that spike initiation and peripheral nociceptive conduction are at least partly functional in spite of the absence of Na\textsubscript{v}1.7. Likewise, it has recently been shown in a CIP patient and Na\textsubscript{v}1.7 conditional knock-out animals that naloxone can partially restore pain [38]. This means that at least part of the peripheral input must reach the presynapse, but due to synaptic inhibition induced by an upregulated opioidergic system in Na\textsubscript{v}1.7 deficient animals/humans is not transmitted further centrally. However, for the olfactory system of
Na_{v}1.7-deficient animals transmission failure has been shown, action potentials properly
generated in sensory neurons do not initiate synaptic signaling [58].

These studies indicate that peripheral somatic nerves may still generate action potentials and
propagate, at least part of them, centrally in the absence of Na_{v}1.7. In the light of the
biophysical properties of Na_{v}1.7, its absence would be expected to have an influence on
initiated conduction of action potentials in peripheral nerves. Its relatively hyperpolarized
activation and inactivation voltage in comparison to Na_{v}1.8 [3;50] and its rapid
activation/inactivation kinetics [30] make it suited as an amplifier of subthreshold
depolarizations, setting the spike threshold. Furthermore, its slow repriming [24], its
prominently slow closed-state inactivation and recovery [14] and the ability to generate
resurgent currents [18] are indicative of determining discharge patterns and neural
accommodation.

So far, studies on pain pathways in Na_{v}1.7 global [21] or conditional knock-out mice
[36;37;40] focus on pain behavior or DRG discharge or currents. Here we compare generation
and conduction of action potentials and their accommodation in the peripheral sensory
endings and nerves of DRG-selective (Advillin/Cre) SCN9A knock-out and wild-type mice.
Apart from general physiological interest, for development of drugs targeting Na_{v}1.7 it is
essential to know whether a compound should engage the neuron before or behind the blood-
brain barrier.
MATERIALS AND METHODS

Chemicals and solutions

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

Animals

The Advillin-Cre transgenic mice, were a generous gift from J. N. Wood [37]. Floxed SCN9AloxP/loxP mice were kindly supplied by T. Leinders-Zufall [58]. The Naᵥ1.7 gene was selectively deleted in sensory neurons expressing Advillin, by crossing Advillin-Cre with SCN9AloxP/loxP mice [37]. Genomic DNA from the tails of KO mice was genotyped using the polymerase chain reaction (PCR). The following primers were used (Invitrogen) for Advillin-Cre genotyping: primer1 (Adv forward) - CCCTGTTCACTGTGAG TAGG, primer2 (Adv WT-reverse) – AGTATCTGGTAGGTGCTTCCAG and primer3 (Adv-Cre reverse) - GCGATCCCTGAACATGTCCATC. Following a 3 min period at 96°C, DNA samples were denaturated (96°C 30sec), annealed (63°C, 30sec) and extended (72°C, 1min) for 30 cycles with a subsequent 10 min period at 72°C. The wildtype band from Primers 1+2 was 480 bp long and mutant allele from Primers 1+3 was 180 bp long. The Primers used for loxP genotyping: primer4 (SCN9A forward) - CAGAGATTCTGCATTAGAATTTGTTC, Primer5 (SCN9A WT/floxed reverse) – AGTCTTTGTGGCACACGTTACCTC and Primer6 (SCN9A KO reverse) - GTTCTCTCTTTGAATGCTGGGCA. Following a 2 min period at
94°C, DNA samples were amplified (94°C 30sec), annealed (60°C, 30sec) and extended (72°C, 2min) for 34 cycles. Wildtype band from primers 4+5 was 317 bp long, loxP allele from primers 4+5 was 461 bp long and a NaV1.7 KO allele was indicated by a 395 bp long band from Primers 4+6. Both Advillin-Cre and SCN9A<sup>loxP/loxP</sup> mice were continuously crossed with C57BL6 to conegnity so C57BL/6 mice were used as control animals [58]. Inbred C57BL/6 and NaV1.7<sup>Adv</sup> conditional KO mice of both sexes and ranging in weight between 20-25g were housed in group cages in a temperature-controlled environment with a 12h light-dark cycle and were supplied with food and water <i>ad libitum</i>. Animals were killed in a rising CO<sub>2</sub> atmosphere in accord with German and European laws.

**Transcriptional regulation**

Total RNA was extracted from homogenized dorsal root ganglia (10-20 ganglia per mouse, 3 mice from each genotype) with the RNeasy Mini kit and treated with DNaseI (QIAGEN, Hildcn, Germany). First-strand cDNA was synthesized from 150 ng oligo(dT)-primed RNA with the RevertAid Reverse Transcriptase kit from Thermo Fisher Scientific. For quantitative real-time RT-PCR (qRT-PCR) analysis, cDNA reactions were mixed with 2x Absolute QPCR Mix, SYBR Green, Rox Mix (Thermo Fisher Scientific), the appropriate primers (from Thermo Fisher Scientific) and filled with water to 20 µl. NaV1.7 Primer sequences are listed above under “Animal” section, otherwise, the following Primers were used (all in 5’ → 3’ direction): ACTB qRT-PCR forward – CGGTCCGGATGCATATGCCGCTTCTTT, ACTB qRT-PCR reverse – CGTCACACTTCATGATGGAATTGA. Expected product: 100 bp. SCN8A qRT-PCR forward - CGTACTATTTGACGCAAAAAACTT. SCN8A qRT-PCR reverse – TCATGCTGAAGACTGAATGTATCA. Expected product: 153 bp. SCN9A qRT-PCR forward – GCCTTGTTTCGGCTAATGAC, SCN9A qRT-PCR reverse – TCCCAGAAATATCACCACGAC. Expected product: 111 bp. SCN10A qRT-PCR forward –
GTGTGCATGACCCGAACTGAT, SCN10A qRT-PCR reverse – CAAAACCCTCTTGCCAGTATCT. Expected product: 101 bp. SCN11A qRT-PCR forward – CCCCTGACCTTATAGCGAAGC, SCN11A qRT-PCR reverse – CTCTTGGCGCTGAAGCGATA. Expected product: 112 bp. Primers were initially tested at concentrations of 100, 300 and 900 nM and finally used at 300 nM. qPCRs including no-template and reverse transcriptase-minus controls were performed in triplicates in an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) with 15 minutes as initial stage at 95°C to activate the DNA polymerase, followed by 40 PCR cycles of 95°C for 15 sec and 60°C for 1 minute. Dissociation curves were generated by heating to 95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec. PCR products were additionally separated on agarose gels and visualized by ethidium bromide staining. Standardization was performed by quantification of the beta Actin gene as an endogenous control and the $\Delta\Delta$Ct method. A cut-off of above twofold increase/decrease was set as a criterion for changes in gene expression. Statistical analysis was performed on at least three independent experiments (i.e., mice) and results are shown as fold increase in Na$_V$1.7 KO mice vs. C57BL6 controls.

Behavioral tests

All animal experiments were approved by the responsible appropriate Animal Protection Authority (Regierung von Unterfranken, Würzburg, Germany). Paw withdrawal latency measurements were performed on both male and female 8-10 weeks old mice. All mice were tested in a "blind" manner, with the person performing the measurements unaware of the mouse genotype. After acclimatization to the testing environment (a few hours exposure to the experimental environment in the two consecutive days prior to the experiment), mice were subjected to repeated plantar mechanical and heat stimulation. Mechanical threshold of the
paw was determined using a dynamic plantar aesthesiometer (Ugo Basile, Gemonio, Italy). A movable unit supplied with a von Frey filament (0.5mm diameter) applied linearly increasing forces onto the plantar side of the hindpaw until withdrawal, at which point the threshold force was recorded. Measurements were done on alternating feet (at least 6 times per mouse), with at least 3 min pause between each stimulus, and results from each mouse were averaged.

For thermal stimulation, an infrared light beam was focused to the plantar side of the hindpaw (Ugo Basile, Gemonio, Italy) through a grid floor on which the mice were allowed to walk unrestricted. The beam was automatically switched off upon withdrawal of the paw, recording the latency. Both left and right paw were measured alternately (at least 8 measurements per mouse) with minimum 3 minutes between stimulations. Two radiant heat stimuli of different intensity (7 and 9 in arbitrary units) were alternately applied and latencies for each intensity were averaged. The total number of sensory stimuli per animal (mechanical + thermal) was between 14 and 20.

**Single-fiber electrophysiology**

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

Mechanosensitive receptive fields were mapped using a blunt glass rod. Once a distinct receptive field was identified, electrostimulation was applied to the RF through a metal microelectrode to determine the fiber’s conduction velocity. Values of conduction velocity were used for classification with a cut-off criterion of <1.4 m/s for unmyelinated (C) fibers [6]. Nav1.7 is primarily expressed in small DRGs and unmyelinated nerve fibers [7;46] thus, electrophysiological recordings in this study were focused solely on unmyelinated C fibers. Lack of initial spontaneous activity was a prerequisite for further testing of all units. A
marking technique was applied, in which latency shifts are provoked through simultaneous application of mechanical and electrical stimuli to the RF [48], ensuring recording from a distinct single-fiber. To assess sensory properties, the mechanical threshold of fibers was characterized using calibrated von Frey (polyamide) bristles ranging in force from 1-128 mN in a geometric scale and equipped with varnished tips (Ø 0.8mm). Subsequent to characterisation, a metal ring (9 mm diameter) was placed encircling the RF. Vaseline was applied to the base of the ring to improve fluid isolation. The fluid volume within the ring was replaced by ice-cold buffer for noxious cold stimulation, or heated by a thermode coupled to a custom-made Peltier device for noxious heat stimulation (20 s ramp of 32 ° - 46 °C). Each stimulus was followed by a resting period of several minutes prior to onset of the following stimulus to minimize the risk of sensitizing effects between modalities. Fibers were considered cold/heat responsive if they produced at least 2 spikes concurrent to stimulation onset. Heat threshold was defined as the temperature at which the second spike of the heat response occurred.

The electrical excitability of the cutaneous nerve endings was determined as the voltage threshold for eliciting an action potential. Rectangular constant voltage stimuli of variable width (in ms: 0.02, 0.2, 2, 20, 200) were applied to the most mechanosensitive spot within the receptive field. Activity-dependent slowing (ADS) of conduction velocity was measured during an electrical stimulation protocol consisting of a 3 min pause, 6 min 0.25 Hz, 3 min 2 Hz, 6 min 0.25 Hz [20]. Changes in conduction velocity are expressed as percentage of initial values (at stimulation onset) of the individual nerve. Maximal slowing is calculated for the last 30 sec of the 2 Hz stimulation. In addition, a recovery cycle protocol at 0.2ms and supramaximal strength was performed, [9;57]. In a continuous stimulation frequency of 0.5 Hz single conditioning pre-pulses at different inter-stimulus intervals (6-500 ms) were
interposed and the latency shift of the conditioned action potential as compared to its conditioning predecessor was analyzed. Finally, some of the fibers were electrically challenged at the RF with ongoing paired electrical pulses (20 ms intrastimulus intervals) at 2 Hz [20;56] and 2-3 fold individual threshold voltage (increased if necessary to compensate for a possible drift of electrical threshold). This provides the time required to provoke conduction block as an index of conduction reliability. Conduction block was defined as the condition when the paired pulses ceased to yield two discernible spikes.

**Compound action potential recordings**

Compound action potential (CAP) signals were recorded extracellularly from isolated segments of mouse saphenous nerve. The saphenous nerves from C57BL/6 and NaV1.7Adv knock-out mice were dissected bilaterally from their point of leaving the inguinal region to approximately 5mm below the knee. Nerves were desheathed to remove the epi- and perineurium and placed in a recording chamber between two suction electrodes. The bath was perfused continuously with SIF, bubbled continuously with carbogen (95% O$_2$: 5%CO$_2$) to a pH of 7.4. Experiments were performed in room temperature as at this temperature the superimposed action potential was still above detection level in all nerves. For electrical stimulation and recording the cut ends of the nerve were pulled through a silicon membrane, creating an optimal seal of the recording and stimulating neuronal sites. The chamber is a modification of the previously published one [12]. Silver wire electrode served as the cathode and anode for stimulation for one suction electrode and for a differential recording at the other. CAP responses were evoked using constant current stimulation (A395, WPI, Sarasota, USA) of fixed duration (1 ms) For the determination of amplitude and conduction velocity (i.e. conduction latency over the fixed recording distance of 5 mm between electrodes) the C-fibre CAP response to supra-maximal electrical stimulation was assessed. To elucidate CAP
changes in response to a repetitive stimulation challenge, a stimulus protocol was used
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
s). Changes in conduction latency and CAP amplitude during this protocol are expressed as
percentage change of their initial value at stimulation onset, i.e. relative to the first value after
the 3 minute pause.

CGRP release

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

Statistics

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

Behavioural tests in naive animals

Behavioral tests in animals represent the integrative performance of both central and peripheral nervous systems. We tested the mechanical and heat sensitivity of Na\textsubscript{v}1.7\textsuperscript{Adv} conditional KO vs. congenic control mice. From their general appearance, control animals could not be differentiated from KOs, which were as heavy, big, groomed and vigilant as controls. By a blinded examiner, two heat stimulus intensities were tested in each group, both revealing a significantly longer withdrawal latency in Na\textsubscript{v}1.7\textsuperscript{Adv} KOs (Fig. 1A): 27.4 ± 3.3 sec for the lower stimulus intensity (7 arbitrary units) and 25.5 ± 3 sec for the higher intensity (9 arbitrary units), versus 14 ± 2.6 sec and 12.6 ± 2.8 sec, respectively, in controls (p<0.05 U-Test). Though less substantial, the mechanical threshold (dynamic von Frey) was also significantly elevated in KOs (Fig. 1B): 4.2 ± 0.7 mN versus 3.1 ± 0.6 mN in controls (p<0.05 U-Test).

Recordings from unmyelinated cutaneous single-fibers

The lack of Na\textsubscript{v}1.7 in peripheral sensory neurons resulted in reduced behavioural noxious heat and mechanical responsiveness of mice. We therefore tested whether this deficiency would be recapitulated in the properties of the cutaneous nerve endings. 60 mechanosensitive cutaneous C-units from controls and 30 from Na\textsubscript{v}1.7\textsuperscript{Adv} KOs were recorded. The fiber population from controls comprised (Fig. 2A): 45 mechano-heat sensitive C-fibers (CMH; 75%), 10 C high threshold mechanosensitive fibers (C-HTM; 17%), 2 low-threshold mechanosensitive C-fibers (C-LTM; 3%), 2 mechano-cold sensitive C-fibers (CMC; 3%) and 1 mechano-heat-cold C-fiber (CMHC; 2%). The fiber population in Na\textsubscript{v}1.7\textsuperscript{Adv} KO was significantly different from that of controls, with mechano-heat sensitive fibers no longer being the most abundant ($\chi^2$ (df
= 4, n= 90) = 15, p< 0.001): 10 CMH fibers (33%), 13 C-HTM fibers (43%), 4 CMC (13%), 2
C-LTM (7%) and 1 CMHC (3%). The sensory capacities (mechanical and thermal) of the
fiber populations from both genotypes were compared. The mechanosensitivity of the KO
units did not significantly differ from that of controls (Fig. 2B-C). The 10 \( \text{Na}_1.7^{\text{Adv}} \) KO C-
fibers that were heat sensitive surprisingly seemed more responsive to heat than the control
units (with higher discharge rate during stimulation, though not significant, Fig. 2D) and a
lower mean heat threshold in comparison to controls (37.9 ± 1.5 °C and 40.3 ± 0.4 °C,
respectively, Fig. 2E, p=0.059, U-Test).

We next examined the conductive properties of the single units. The mean conduction
velocity of KO fibers was significantly lower than that of controls by about 20%, while the
variance was about the same (Fig 3A-B, 0.4 ± 0.04 m/s vs. 0.5 ± 0.02 m/s, respectively
(p<0.05 U-Test). The single-fiber recordings from \( \text{Na}_1.7 \) KO mice have not shown reduced
sensitivities to the diverse modalities of mechanical and heat stimulation. A possible
explanation for that would be that the voltage threshold for triggering propagated action
potentials in the skin nerve endings is about the same in KOs and WTs. We therefore assessed
the electrical excitability, placing an electrode (cathode) in the receptive field at the spot of
highest mechanosensitivity. In addition to varying the stimulus voltage, we also varied the
stimulus duration in order to gain information about a potential kinetic difference between KO
and WT nerve terminals. However, the resulting strength-duration curves did not significantly
differ between the genotypes (Fig. 3C, left graph, multiple T-tests with Bonferroni
correction). Also the chronaxy did not appear different between KO and WT. This became
evident when the parabolic curve was linearized by plotting the product of threshold voltage
and stimulus duration (an analogue to threshold charge transfer) over stimulus duration in a
linear coordinate system (Fig. 3C right graph). In this representation the chronaxy
corresponds to the abscissa intercept, which is obviously the same in both genotypes, whereas the slightly different slopes of the (regression) lines correspond to the non-significant difference of the rheobases. This means that the kinetic requirements (utilization times) of the electrical stimulus to trigger an action potential are not different between Na\textsubscript{V}1.7--/-- and WT. In other words, Na\textsubscript{V}1.7 is not required as an action potential generator in those C-fibers that retained mechanosensitivity and electrical excitability in the KOs.

The recovery cycle experiment (Fig. 3D) with electrical double pulses at varying inter-stimulus intervals (ISI) studies the impact of a preceding action potential on the conduction velocity (latency) of the subsequent, second, spike. Apart from the prolonged latency (slower conduction velocity) in KOs vs. WTs, visible at longer ISI (> 100 ms), the exponential increase of the latency with shorter ISIs (< 100 ms) in the KO fibers is striking and obviously different from the moderate increase in WT fibers. These changes are attributed to long lasting afterpotentials, most probably of hyperpolarizing direction. Thus, Na\textsubscript{V}1.7 seems to stabilize the conduction velocity at shorter spikes intervals (higher discharge rates).

This role of Na\textsubscript{V}1.7 in stabilizing conduction velocity lead us to examine the activity-dependent slowing of conduction velocity (ADS) upon prolonged low frequency (e.g. 2 Hz) stimulation. This phenomenon is due to progressive slow inactivation of the voltage-gated sodium channels and to accumulation of sodium ions in the thin nerve fibers [15;53]. During a control period of 6 min at 0.25 Hz the fibers became slightly slower in conduction, showing no difference between both genotypes (Fig 4A). During the more frequent electrical stimulation at 2 Hz for 3 min, the ADS became progressively prominent in the WTs, but the KO fibers soon lagged behind and finally reached clearly less maximal ADS than the WT units (Fig. 4B: 35.5 ± 6.2% vs. 57.4 ± 4.7%, respectively, p<0.05, U-Test). During the subsequent recovery period of 6 min at 0.25 Hz both latencies returned towards their original
values. Progressive ADS of single units often lead to conduction failures, in which condition fibers do not reliably respond to each electrical stimulus with a propagated action potential. Our stimulation test was a 4 min period of 2 Hz double-pulse (ISI 20 ms) stimulation. In accord with the finding of less ADS, the KO fibers were much less prone to block in comparison to controls, more reliably responding to the second of the paired stimuli (Fig. 4C, block of 6/13 units as opposed to 12/13, respectively).

In conclusion and comparison to WT fibers, the mechanosensitive cutaneous C-fibers that we could identify in Na\textsubscript{\textit{V}}\textsubscript{1.7}\textsuperscript{Adv} KO mice, exhibited (1) a significantly lower prevalence of responsiveness to moderate noxious heat, although (2) the rarer heat responses seemed to show exceptionally high mean discharge rates and low heat thresholds; (3) mechanosensitivity was about normal. In biophysical respects, (4) conduction velocity was 20% slower and (5) became progressively slower in response to the second of two electrical stimuli applied at short interval (< 100 ms); (6) electrical excitability, rheobase and chronaxy, were about normal. Upon prolonged electrical stimulation at 2 Hz, (7) activity-dependent slowing of nerve fiber conduction was markedly less expressed and (8) more rarely led to conduction failure of the KO single-fibers. Above all, it seems that a subpopulation of moderately heat responsive polymodal nociceptors is, at least functionally missing in Na\textsubscript{\textit{V}}\textsubscript{1.7}\textsuperscript{Adv} mice, while the retained and detectable C-fibers in these mice exhibit anomalous biophysical properties that do not indicate any susceptibility to failure of action potential generation or conduction.

**Stimulus-induced cutaneous CGRP release**

The single-fiber recordings suggested the absence of a subpopulation of heat sensitive nociceptors in Na\textsubscript{\textit{V}}\textsubscript{1.7}\textsuperscript{Adv} KO mice. A way to test the overall heat responsiveness of a skin flap is to measure stimulated CGRP release which can serve as an index for the activation of, at least, the peptidergic neurons [26]. However, hindpaw skin flaps of Na\textsubscript{\textit{V}}\textsubscript{1.7}\textsuperscript{Adv} KO and control
mice revealed no significant difference in CGRP release evoked by heating to 45°C or, for control, by depolarisation with 60mM KCl (Fig. 5). This CGRP release depends on calcium influx through heat-activated ion channels or voltage-gated calcium channels, respectively, but not on action potential generating sodium channels [51]. Thus, the results exclude the absence of a peptidergic subpopulation of fibers, e.g. for possible developmental reasons in the transgenic mice, but they do not exclude the functional inability of these fibers to generate propagated action potentials.

**Compound action potential recordings**

The single-fiber recordings from the saphenous innervation territory suggested both the functional absence of a whole subpopulation of conducting C-fibers and altered conduction properties of the retained C-fibers as a result of the conditional deletion of Na\textsubscript{V}1.7 in the DRGs. Both consequences should show up in whole saphenous nerve recordings of the compound action potentials. Indeed, the A-fiber CAP amplitudes were about the same in KOs and WTs, but the C-CAPs of the KO mice were by two thirds smaller and half as fast in conduction velocity than in the WTs (Fig. 6 A-C). These deficits can hardly be due to dispersion (in time) of the electrically evoked action potentials, as the single-fiber spikes showed about the same amplitudes and even slightly less scattering of the reduced conduction velocities in KOs as compared to WTs (see above). Also the finding of less ADS in KOs than WTs was fully recapitulated in the C-CAP recordings (Fig. 6D).

**Transcriptional regulation**

It has previously been shown that in Na\textsubscript{V}1.8 KO mice there is transcriptional upregulation of Na\textsubscript{V}1.7 mRNA [4], also of relevance for neuronal excitability in whole animals [34;63]. To make sure our results do not stem from similar transcriptional upregulation we have screened...
KOs for expression of specific sodium channels. Quantitative PCR results from DRGs demonstrate that the conditional KOs had no significant change in gene expression of Na\(_v\)1.6, Na\(_v\)1.8 and Na\(_v\)1.9 in comparison to control mice (Fig 7).
DISCUSSION

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

A central question is whether these typical polymodal C-fibers are physically absent in Nav1.7Adv KOs, or just unable to generate/propagate action potentials along their axons? At least one essential function; neuropeptide (CGRP) release from cutaneous sensory axons and terminals was largely unaffected in the KOs. This important function requires the presence of peptidergic nerve fibers connected by axonal transport to their DRG cell bodies, but it does not require action potential discharge, just depolarization and calcium influx are sufficient [51]. The axon reflex erythema upon histamine injection is reported to be functional in human CIP mutants [22]. This suggests that action potentials are generated and (antidromically) conducted through wide branching arborisation of the CGRP-expressing nerve fibers. It does however not mean that these action potentials are propagated up the peripheral nerve.
Our previous studies with pharmacological elimination of Na\textsubscript{V}1.7 by TTX indicate that actually almost all cutaneous C-fibers are more or less affected by the toxin, at least at higher temperatures [63]. This raises the question whether the subpopulation of single-fibers compensating the lack of NaV1.7 by other channels such as NaV 1.8 or 1.9 in our present study show biophysical symptoms resulting from the NaV1.7 deletion. An interplay of the two major players NaV1.7 and NaV1.8 has been made responsible for electrogensis in DRG neurons [44]. While NaV1.7 opens in the subthreshold range and amplifies slow small depolarisations (often together with NaV1.9) NaV1.8 takes over at more depolarised membrane potentials to finally carry most the APs sodium influx ([25], for review see [55]). Rapid repriming and relative resistance to inactivation make NaV1.8 also responsible for conduction safety [27]. However this interplay depends on cell type. Rush and colleagues have shown, that an erythermalgia gain of function mutation of NaV1.7 (L858H) may result in hyperexcitability of DRG neurons but hypoexcitability of sympathetic neurons [45]. Likewise neurons – or even sections of one neuron (soma, axon, terminal) - with different expression patterns or availability patterns (i.e. inactivity patterns at various resting membrane potentials) of sodium channels (NaV1.8 in the above example) might modulate this interplay. Changes of electrogensis in the axon is expected to have an influence on conduction velocity. In modelling studies conduction velocity (of myelinated fibres) has been predicted to be mainly influenced by sodium channel kinetics i.e. composition of kinetically different subtypes is more important than overall channel density [39]. Indeed, we found conduction velocity of NaV1.7 deficient axons to be significantly lower by 20% than in WT fibers. Using the same methods, we could not see altered conduction velocity in NaV1.8 [27] or NaV1.9 [26] knock out animals. However unmyelinated axons lacking NaV1.6 [8] also slow down conduction of compound action potentials by ~30%. A lack of the persistent current of NaV1.6
might have hyperpolarised these axons and thereby slowed down conduction. Also our results suggest hyperpolarisation of $\text{Na}_V 1.7$ deficient terminals. The recovery cycle experiments revealed a pronounced sub-normality at double-pulse intervals shorter than 100 ms in the KOs, much less in the WTs. This is reminiscent of the exceptionally long after-hyperpolarizations, up to 100 ms, that DRG neurons with nociceptive C-fibers show [32].

Indeed, the conduction velocity of the units was significantly lower by 20% than in WT fibers. The recovery cycle experiments revealed a pronounced sub-normality at double-pulse intervals shorter than 100 ms in the KOs, much less in the WTs. This is reminiscent of the exceptionally long after-hyperpolarizations, up to 100 ms, that DRG neurons with nociceptive C-fibers show [32]. If this finding also applies to nerve endings, $\text{Na}_V 1.7$ could rapidly recover from fast voltage-dependent inactivation during the AHP and quickly respond to the subsequent electrical stimulus, due to its relatively hyperpolarized activation range. If, however, $\text{Na}_V 1.8$ has to initiate the action potential, as in the KOs, it will take longer to reach its more depolarized activation voltage range starting from a still hyperpolarized membrane potential and, accordingly, the conduction velocity will drop. The underlying difference in voltage-dependent activation between $\text{Na}_V 1.7$ and $\text{Na}_V 1.8$ is well established in DRG neurons [16]. However, it does not seem to apply to the unmyelinated nerve endings in the skin, as the parameters of electrical excitability, rheobase and chronaxy, were not significantly different between $\text{Na}_V 1.7$ Adv KOs and WTs. This should not be due to an insufficient power of resolution of the extracellular electrostimulation technique, because the expectable reduction of excitability in global $\text{Na}_V 1.9$ KOs could recently be demonstrated using the same threshold tracking technique [26]. Thus, the reason for the discrepancy between DRG cell bodies and their cutaneous terminals remains unclear but may relate to the fundamental difference in membrane surface – to – volume ratio.
The experiments on activity-dependent slowing of the conduction velocity refer to a different time scale than the recovery cycle trials, the interstimulus interval was 500 ms and the challenge of the C-fibers by continuous electrical stimulation lasted for 3 min. During this period the ADS in WTs finally reached a plateau which was significantly lower in the KOs. Two parallel mechanisms seem to account for the ADS phenomenon, an accumulation of slow voltage-dependent inactivation of the sodium channels and an accumulation of sodium ions inside the thin axons which reduces their inward driving force [15,53]. We tend to assume that the absence of the Na\textsubscript{V}1.7-carried sodium current, reducing the accumulation, is a reason for less ADS in the KOs. Both mechanisms can obviously lead to conduction failures, in particular at branch points. This affected every single one of the WT fibers but only half of the KO units during a 4 min challenge with 2 Hz double pulses. Again, we assume that less sodium accumulation is responsible for this increased safety factor of conduction in the KOs.

We found many functional nociceptive C-fibers in the saphenous of Na\textsubscript{V}1.7\textsuperscript{Adv} KOs but also an indication that one heat sensitive subpopulation of units was reduced, either physically absent or unable to conduct action potentials. Our compound action potential recordings cannot decide between these alternatives, but they suggest that a subpopulation of C-fibers is not conducting at normal peripheral skin temperature (32°C). A principal problem with CAPs is that their amplitude can shrink either by enhanced dispersion of the individual fiber conduction velocities or by reduced size or number of the superimposed action potentials. However, the single-fiber recordings did not reveal a greater scattering of the spike latencies in the KOs and the spike amplitudes appeared normal with respect to the signal-to-noise ratio. The conduction velocities were slower than in WTs, but this was also reflected by the C-CAP which was equally slower in conduction in the KOs. Thus, most probably, it is the number of
superimposed C-fiber APs that is reduced in the KOs, in contrast to the A-fibers, for which A-CAPS were not different between KO and WTs.

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

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FIGURES/LEGENDS

Fig. 1: Behavioural tests in conditional Na\textsubscript{\textit{v}}\textsuperscript{1.7\textsuperscript{Adv}} KO vs. congenic WT control mice. A. Blind testing of radiant heat-induced hindpaw withdrawal (Hargreaves’ test) using two stimulus intensities (7 and 9, arbitrary units). B. Dynamic von Frey testing of mechanical hindpaw withdrawal thresholds. Asterisks indicate p<0.05 using U-test.
Fig. 2: Cutaneous C-fiber populations and sensory properties in Na1.7Adv KOs vs. WTs. A. Sensory categorization of mechanosensitive unmyelinated single-fibers *in vitro* reveals differential prevalence between the genotypes, in particular with respect to heat sensitivity ($\chi^2$ (df = 4, n= 90) = 15, p< 0.001). B. Distribution of dynamic von Frey thresholds in KOs and WTs over a geometric force scale. C. Boxplot of the mechanical thresholds. D. Averaged discharge rates per second in response to a 20 s radiant heat ramp (grey background); large variability in KO fibers due to small sample size. E. Mean heat thresholds of the units (temperature at second spike, U-test).
Fig. 3: Conductive properties of C-fibers in KOs and WTs. A. Distribution of conduction velocities over the distance from cutaneous receptive field to recording electrode. B. Modified boxplot showing mean instead of median; asterisk indicates p<0.05, U-test. C. Strength-duration curves determined by threshold tracking with electrode in the receptive field (left graph). Right graph: The parabolic curves in the left graph are linearized by calculating an analog to threshold charge transfer (V · ms), showing the slopes (i.e. rheobases) of the regression lines and identical abscissa intercepts (i.e. chronaxies). D. Electrical double pulses at varying ISI reveal differential retardation of the second spike, in particular at short intervals (p < 0.05, multiple T-tests with Bonferroni correction).
Fig. 4: Activity-dependent slowing and propagation safety of C-fibers in KOs and WTs.

A. Differential ADS development over time in percent of initial latency. B. Maximal ADS, mean (± SEM) during the last 30 s of the 2 Hz stimulation period, the asterisk indicates p<0.05, U-test. C. Conduction failures during 4 minutes of electrical double pulse (20 ms ISI) stimulation at 2 Hz. The decreasing number of fibers reliably conducting over time is depicted.
**Fig. 5:** Stimulated CGRP release from isolated skin. A. Response to 45°C heat stimulation. B. Response to unspecific depolarisation with 60 mM external KCl.

**Fig. 6:** Compound action potentials of isolated saphenous nerves of KOs and WTs. A. Single nerve examples showing fused electrical artefact and A-fiber CAPs as well as C-CAPs of differential latency and amplitude at 32°C. B. Conduction velocity of C-Caps, the asterisk indicates p<0.05, U-test. C. Peak-to-peak amplitudes of C- and A-CAPs, the asterisk indicates p<0.05, U-test. D. Development of ADS of the C-CAPs.
Fig. 7: Gene expression in DRGs from Na\textsubscript{v}1.7 KO mice. Results are shown as fold change of gene expression in KOs vs. control mice.
Reference List


[32] Lawson SN. Phenotype and function of somatic primary afferent nociceptive neurones with C-, Adelta- or Aalpha/beta-fibres. Exp Physiol 2002;87:239-244.


[51] Spitzer MJ, Reeh PW, Sauer SK. Mechanisms of potassium- and capsaicin-induced axonal calcitonin gene-related peptide release: involvement of L- and T-
type calcium channels and TRPV1 but not sodium channels. Neuroscience 2008;151:836-842.


