

Na_v1.7 is required for normal C-low threshold mechanoreceptor function in humans and mice

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13 **Running title:** The role $\text{Na}_v1.7$ in C-LTMRs

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ACCEPTED MANUSCRIPT

1 **Abstract**

2 Patients with bi-allelic loss of function mutations in the voltage-gated sodium channel Nav1.7
3 present with congenital insensitivity to pain (CIP), whilst low threshold mechanosensation is
4 reportedly normal. Using psychophysics ($n = 6$ CIP participants and $n = 86$ healthy controls) and
5 facial EMG ($n = 3$ CIP participants and $n = 8$ healthy controls) we have found that these patients
6 also have abnormalities in the encoding of affective touch which is mediated by the specialised
7 afferents; C-low threshold mechanoreceptors (C-LTMRs). In the mouse we found that C-LTMRs
8 express high levels of Nav1.7. Genetic loss or selective pharmacological inhibition of Nav1.7 in
9 C-LTMRs resulted in a significant reduction in the total sodium current density, an increased
10 mechanical threshold and reduced sensitivity to non-noxious cooling. The behavioural
11 consequence of loss of Nav1.7 in C-LTMRs in mice was an elevation in the von Frey mechanical
12 threshold and less sensitivity to cooling on a thermal gradient. Nav1.7 is therefore not only
13 essential for normal pain perception but also for normal C-LTMR function, cool sensitivity and
14 affective touch.

15
16 **Keywords:** affective touch; C-low threshold mechanoreceptors; congenital insensitivity to pain;
17 Nav_v1.7

18 **Abbreviations:** C-LTMRs = C-low threshold mechanoreceptors; CIP = congenital insensitivity to
19 pain; DRG = dorsal root ganglion; EMG = electromyography; GOF = gain of function; HSAN =
20 hereditary sensory and autonomic neuropathy; IHC = immunohistochemistry; KO = knock-out;
21 LOF = loss of function; PEPD = paroxysmal extreme pain disorder; TH = Tyrosine hydroxylase;
22 VGSCs = voltage-gated sodium channels; VAS = visual analogue scale

23

1 Introduction

2 Touch sensation is a critical component of the sensory system giving us the ability to detect,
3 discriminate and explore our environment and also provides a substrate for social interaction.
4 Low threshold mechanoreceptors (LTMRs) are a heterogenous group of sensory neurons which
5 encode mechanical stimuli and can be classified according to their conduction velocity, stimulus-
6 response function and the end-organs which they innervate. Although the majority of C-fibre
7 sensory afferents are nociceptors and thermoceptors, an unmyelinated C-fibre population, termed
8 C-low threshold mechanoreceptors (C-LTMRs, Often termed CT- afferents in humans) were first
9 discovered 80 years ago in the cat¹ and much later in humans.^{2,3} In humans, C-LTMRs respond
10 to low threshold punctate indentations and have conduction velocities in the C-Fibre range.³
11 Human C-LTMRs also underpin pleasant touch, a category of tactile perception which until
12 recently has been largely understudied. Yet evidence now suggests they are highly important for
13 social contact, communication, relationships, pain relief, and empathy for touch observed in
14 others.⁴⁻⁶ These afferents respond to brushing stimuli between 1-10 cm s⁻¹ and show an inverted
15 U-shape relationship, with peak firing rates and peak perceived pleasantness seen at 3 cm s⁻¹.⁷ A
16 sub-set of these afferents have also been shown to respond to cooling stimuli in human.²

17 C-LTMRs have been identified in other mammals, including rodents.^{8,9} Several studies
18 have identified molecular markers of rodent C-LTMRs (vGLUT3¹⁰, Tafa4¹¹ and IB4-GINIP+¹²),
19 one of which being Tyrosine hydroxylase (TH), a marker of all C-LTMRs.¹³ In addition, recent
20 single cell RNA sequencing studies have provided further evidence confirming that C-LTMRs
21 have a very distinct transcriptional profile compared to other sensory neuron populations.^{14,15}
22 The TH positive population makes up ~10% of all dorsal root ganglion (DRG) neurons and
23 sparse genetic labelling of TH positive C-LTMRs revealed sensory endings innervating mouse
24 hairy skin as longitudinal lanceolate endings surrounding hair follicles.¹³ C-LTMR sensory
25 neurons also project centrally to the spinal cord and terminate in lamina Iii of the dorsal horn
26 (DH) where they synapse with distinct inhibitory (parvalbumin) and excitatory (PKC γ)
27 interneuron populations.^{13,16} Li *et al* (2011) also confirmed that TH positive DRG neurons
28 function like human C-LTMRs and have low mechanical thresholds, C-fibre range conduction
29 velocities and respond to cooling stimuli. Because molecular identifiers of C-LTMRs have
30 emerged relatively recently, there has been a dearth of studies into the role of sodium channel

1 genes and their mutations in C-LTMRs, in either humans or rodents. This is particularly relevant
2 as voltage-gated sodium channels (VGSCs) have emerged as important analgesic drug targets.
3 Two recent studies suggest that C-LTMRs show significant expression of *SCN9A*^{15,17}, the gene
4 encoding the VGSC Nav1.7 which human genetics has strongly linked to nociception and pain.¹⁸
5 Gain of function (GOF) mutations in Nav1.7 can result in painful conditions such as
6 erythromelalgia, paroxysmal extreme pain disorder (PEPD), small fibre neuropathy and painful
7 diabetic neuropathy.^{19–23} Whilst bi-allelic loss of function (LOF) mutations lead to congenital
8 insensitivity to pain (CIP) in which patients do not perceive pain in response to noxious
9 mechanical, thermal or chemical stimuli.^{24–26} These striking psychophysical features are
10 accompanied by a loss of functional C-nociceptors (assessed using microneurography)
11 highlighting Nav1.7 as an important modulator of the nociceptive system.²⁷ This sensory loss has
12 been thought to be relatively selective with an absence of pain perception, chemogenic itch and
13 smell; touch and proprioceptive function were reportedly normal.^{24–27} This human genetic data,
14 the relatively selective expression of Nav1.7 in the peripheral versus central nervous system and
15 preclinical studies have made Nav1.7 an attractive druggable target to treat painful conditions.^{28–}
16 ³² A number of small molecule blockers targeting Nav1.7 are currently in clinical development.

17 Whilst understandably there has been a focus linking Nav1.7 to human nociception, C-
18 LTMR function, has never been specifically interrogated. We have used a multi-disciplinary
19 approach to answer this question by studying humans with LOF mutations in Nav1.7 alongside
20 mice in which Nav1.7 has been ablated in C-LTMRs. We find that Nav1.7 LOF in the human not
21 only leads to CIP, but also an impairment in affective touch and that the stimulus-response
22 function of C-LTMRs to mechanical and cooling stimuli is critically dependent on functional
23 Nav1.7. Finally, we challenge the current dogma that therapeutics targeting Nav1.7 will act only
24 on the nociceptive system and that treatments may have undesired impacts on affective touch
25 sensation.

26 **Materials and Methods**

27 **Humans**

28 Six participants with bi-allelic loss of function mutations in *SCN9A* and congenital insensitivity to
29 pain (CIP, 2 males and 4 females, mean age = 35, SD = 11.02, Supplementary Table 1)

1 participated in the psychophysical testing and were compared to a large normative sample of
2 healthy subjects^{4,33,34}, which included age and sex matched controls ($n = 86$, 45 females, age
3 range = 16 - 60, mean age = 36, SD = 12.2). All of the participants exhibited the typical features
4 of congenital insensitivity to pain with a history of never having experienced pain and multiple
5 painless injuries such as burns and fractures. Facial electromyography (facial EMG) was
6 collected in three participants (participants 2, 3, and 4) and 8 age matched controls. Ethical
7 approval was obtained by the ethics board of Linköping University (dnr 2014/341-31, dnr
8 2017/392-32, 2018/623-32 and 2017/485-31) and the National Research Ethics of the United
9 Kingdom (Painful Channelopathies Study, NRES-UK reference: 12/LO/0017). Participants gave
10 informed consent in accordance with the Declaration of Helsinki. See Supplementary material
11 for detailed information on human participants.

12 **Affective touch testing: psychophysics**

13 Single brush strokes were manually delivered to a 9 cm section of the forearm of the
14 participants ($n = 6$ CIP participants and $n = 86$ healthy controls) using a soft 7 cm-wide brush.
15 Thirty brush strokes were delivered in a distal to proximal direction at 5 different velocities in
16 pseudo-randomized order: 0.3 cm s^{-1} , 1 cm s^{-1} , 3 cm s^{-1} , 10 cm s^{-1} , and 30 cm s^{-1} . Participants
17 rated unpleasantness or pleasantness using visual-analogue scale (VAS) with the anchor points
18 'unpleasant' (-10) and 'pleasant' (+10). See Supplementary material for detailed information on
19 psychophysics testing.

20 Average scores per velocity per participant were entered in a 2x5 factorial ANOVA with
21 "speed" as and "group" as factors. In addition, for the healthy control participants, we assessed
22 potential sex differences in a 2x5 factorial ANOVA with "speed" as and "sex" as factors. Post-
23 hoc analysis was performed using Mann-Whitney. All data was analyzed using the Statistical
24 Package for the Social Sciences (SPSS Inc., Chicago, IL, USA).

25 **Affective touch testing: facial electromyography (facial EMG)**

26 Participants (CIP participants 2, 3, and 4, $n = 8$ age and sex matched healthy controls) were
27 fitted with surface electrodes placed above the eyebrow, to measure corrugator supercilii

1 (“corrugator”) muscle region activity, and over the cheek, measuring zygomaticus major
2 (“zygomatic”) muscle region reactivity according to Fridlund and Cacioppo *et al* (1986).³⁵
3 Affective responses were assessed by measuring corrugator and zygomatic reactivity in
4 response to each stimulus, quantified as mean EMG activation during the 6 sec stimulus
5 presentation minus the mean EMG activation during the 1 sec before the stimulus was
6 presented. Touch was administered using a soft 5 cm-wide brush applied to a 9 cm section of
7 the forearm, as detailed previously.^{36,37} The task consisted of four blocks; each block consisted
8 of 8 trials, four at each velocity, 3 cm s⁻¹ (slow) and 30 cm s⁻¹ (fast), with velocity order within
9 each block pseudorandomized but not repeated more than 3 times. During the inter-trial
10 intervals, participants rated on a VAS scale “How PLEASANT was the touch?” or “How INTENSE
11 was the touch?” and could choose from -10 (extremely unpleasant) to +10 (extremely pleasant)
12 or -10 (not at all intense) to +10 (extremely intense), respectively, using the mouse to move the
13 VAS slider. Within-group repeated measures ANOVAs with “speed” as factor were performed
14 on behavioral ratings and on facial muscle activity scores. Individual CIP participant’s scores
15 were compared using independent-samples t-tests or Mann-Whitney when data was not
16 normally distributed. See Supplementary material for detailed information on facial EMG
17 testing.

18 **Animals**

19 All mice were group-housed in individually ventilated cages with free access to food and water,
20 in humidity and temperature controlled rooms with a 12hr light-dark cycle, in a pathogen free
21 facility. All animal procedures adhered to the UK Home Office (Scientific Procedures) Act (1986)
22 and performed under a UK Home Office Project Licence. All animal experiments were carried
23 out in accordance with University of Oxford Policy on the Use of Animals in Scientific Research.
24 The work within this study also conforms to the ARRIVE guidelines.³⁸ See Supplementary
25 material for detailed information on mouse strains used.

26

1 **Immunohistochemistry (IHC) and In-situ hybridisation (ISH)**

2 Animals were deeply anaesthetised with pentobarbital, and the blood cleared from all tissues by
3 perfusing saline through the vascular system. Mice were then perfuse-fixed using 4%
4 paraformaldehyde (PFA). Tissues were then collected and post-fixed in 4% PFA accordingly
5 (DRG: 1-2hrs, Spinal Cord: 24hrs, Skin: 1-2hrs). All tissues were cryoprotected in 30% Sucrose
6 for a minimum of 48hrs followed by embedding the tissue and sectioning on a cryostat. (DRG
7 12µm, Spinal cord 20µm, Skin 30µm). Cultured cells were fixed with 4% PFA for 10 mins and
8 treated similarly to other tissues. Standard IHC protocols were used.

9 ISH was performed using two methods; the first method (related to Fig. 3 and
10 Supplementary Fig. 3C-D) was performed by following the user instructions for the
11 RNAScope2.5 RED Chromogenic assay kit (Advanced Cell Diagnostics), with a Nav1.7mRNA
12 specific probe (Cat no. 457641). The second method of ISH (related to Supplementary Fig. 3B)
13 was performed using digoxigenin labelled probes. Nav1.7 probes were hybridized overnight at
14 55°C, and the slides incubated with the horseradish peroxidase anti-digoxigenin antibody
15 (Roche). Final detection was achieved using cy3 TSA plus kit (Perkin Elmer). (Detailed methods
16 of IHC, ISH and analysis see Supplementary material).

17 **Animal Behaviour**

18 Both male and female mice were used in this study and mice were tested at a consistent time
19 of day, in the same environment by the same experimenter. Mice were habituated to their
20 testing environment and equipment prior to behavioural test days. The experimenter was blind
21 to animal genotype until after behavioural analysis was complete.

22 **Mechanical Sensory testing**

23 Mice were randomly assigned a test box (5x5x10cm) which was elevated on a wire mesh base
24 and were acclimatised to the equipment for 30-60mins. The Plantar hind paws were tested
25 using punctate von Frey hairs, brush and cotton swab stimuli. The dorsum of the hind paws was

1 tested with sticky tape. Tactile acuity was assessed using the sandpaper tactile acuity test. See
2 Supplementary material for details.

3 **Thermal Sensory testing**

4 Mice were randomly selected from their home cages in order to randomise the order of
5 thermal sensory assessment. Noxious thermal sensitivity was assessed using a 53°C
6 hotplate and thermal preference was assessed using a thermal gradient apparatus (54°C to
7 6°C). For further details see Supplementary material.

8 **Whole-cell patch clamp recordings**

9 Voltage-clamp recordings using an Axopatch 200B amplifier and Digidata 1550 acquisition
10 system (Molecular Devices) were performed at room temperature (21°C). Data were sampled
11 at 20 kHz and low-pass filtered at 5 kHz. Series resistance was compensated 80%–90% to
12 reduce voltage errors. All data were analyzed by Clampfit 10 software (Molecular Devices).
13 GFP/eYFP+ DRG neurons (for cell culture methods see supplementary methods) were detected
14 with an Olympus microscope with an inbuilt GFP filter set (470/40x excitation filter, dichroic LP
15 495 mirror and 525/50 emission filter).

16 The protocol used (for solutions see supplementary methods) to assess peak voltage-
17 gated Na⁺ currents consisted of a 20ms test pulse to 0mV, from a holding potential of -120mV.
18 The protocol used to assess the effect of PF-05089771 on Na⁺ currents consisted of a step from
19 -120mV to -75mV for 8s to inactivate a proportion of Nav1.7 channels, followed by a recovery
20 step to -120mV for 2ms and a test pulse to 0mV for 20ms. This inactivation step was necessary
21 as PF-05089771 blocks Nav1.7 by binding in the inactivated state. We were guided by previous
22 literature that -77mV is the half inactivation of hNav1.7.³⁹ In both instances, three sweeps were
23 taken, with an intersweep interval of 10s and the peak inward current during the test pulse was
24 measured for each recording. I/V curves were generated from a series of incremental (Δ +5mV)
25 300ms voltage steps from -80 to +35mV, evoked every 10 s from a holding potential of -120mV.
26 Recordings were discarded if series resistance > 15 M Ω or deviated by > 20% during the
27 recording. Linear leak subtraction was performed using P/4 leak subtraction.

1 **Ex vivo skin-nerve preparation**

2 The hind paw hairy skin and saphenous nerve was dissected and maintained in the inside-out
3 orientation (Hypodermis face up) in a recording chamber constantly perfused with synthetic
4 interstitial fluid (SIF: 2.0mM CaCl₂, 5.5mM Glucose, 10mM HEPES, 3.5mM KCL, 0.7 mM MgSO₄,
5 123mM NaCl, 1.5mM NaH₂PO₄, 9.5mM Na-gluconate, 7.5mM Sucrose, 1M NaOH; dH₂O) at
6 32°C. The saphenous nerve was isolated using mineral oil (Sigma) in an adjacent chamber, de-
7 sheathed and nerve fibres teased apart and placed onto a silver recording electrode. Single
8 fibre receptive fields were located using a blunt probe, and conduction velocity measured using
9 pulsed supra-threshold electrical currents. C-LTMRs were identified on the basis of two main
10 factors; Conduction velocities below 1.2m/s and v-Frey mechanical thresholds below 5.8mN. All
11 stimuli (for stimuli see supplementary methods) evoked action potentials were visualized using
12 an oscilloscope and recorded using a Powerlab 4.0 system in conjunction with LabChart v7.3
13 software (ADInstruments). The experimenter was blind to animal genotype prior to the
14 experiment until post analysis. For pharmacology, once C-LTMRs were identified in C57BL/6
15 mice, receptive fields were isolated using a metal ring and the skin was stimulated pre and 10-
16 12 mins post application of 10nM of the selective Nav1.7 channel blocker PF-05089771 or
17 vehicle. The experimenter was blind to the treatment group until post analysis. On average,
18 only 1-2 C-LTMRs were found per preparation, each preparation lasted up to 8hrs.

19 **Computational modelling of C-LTMRs**

20 The C-LTMR computational model used was previously described in detail by Zheng *et al* (2019)
21 and was accessed from ModelDB (<https://senselab.med.yale.edu/modeldb/>, accession No.
22 256632).¹⁷ CIP participant mutations from participant 2, 3 and 4 were previously characterised
23 by McDermott *et al* (2019) in a heterologous expression system. The fold change decrease in
24 the mutant Nav1.7 conductance, compared to wild type conductance was calculated. In the C-
25 LTMR model, the maximal Nav1.7 conductance was altered according to the conductance fold
26 change due to each mutation (Supplementary Table 2) to create a new model for each
27 mutation. The model was run in the naïve setting to model healthy controls without Nav1.7

1 mutations. The models applied successive current injections in increments of 1pA in order to
2 assess threshold excitability and 25pA to assess suprathreshold excitability.

3 **Statistical analysis**

4 All data was tested for normality using the D'Agostino-Pearson normality test and the
5 appropriate parametric or non-parametric statistical tests used accordingly. All statistical tests
6 used were two-tailed. Statistical comparisons were made using a Student's t-test or Mann
7 Whitney U-test. In experimental groups in which multiple comparisons were made one way or
8 two-way analysis of variance (ANOVA) tests with appropriate post-hoc tests were performed.
9 All data is represented as mean \pm the standard error of the mean (SEM) unless otherwise
10 stated. Statistical significance is indicated as follows * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The
11 statistical test used is reported in the appropriate figure legend. Graph Pad prism 6 was used to
12 perform statistical tests and graph data. Adobe illustrator CS5 was used to create schematics
13 and medical graphics were obtained from Smart servier free medical art (smart.servier.com).

14 **Data availability**

15 The data that support the findings of this study are available from the corresponding author upon
16 reasonable request.

17 **Results**

18 **CIP participants have an altered affective touch experience**

19 We investigated affective touch in 6 participants with LOF mutations in *SCN9A* which principally
20 results in CIP. Details about the participants who were recruited for this study are outlined in
21 Supplementary Table 1. We presented healthy control participants and CIP participants with an
22 affective touch paradigm (Fig. 1A) in which pleasantness was rated in response to brushing the
23 forearm at different velocities. The self-reported scores from a large sample of healthy
24 participants were consistent with previous literature demonstrating an inverted U-shaped VAS
25 pleasantness pattern with stimulus speed and the optimal brushing speed being 3 cm s^{-1} (Fig.
26 1B).^{4,7,33,34,40} No sex differences were identified in healthy participants (Supplementary Fig. 1).

1 The CIP participant group reported significantly lower pleasantness ratings for slow brushing
2 touch and they do not show the classical U-shaped VAS score response (Fig. 1B and
3 Supplementary Fig. 1). The change in self-reported pleasantness in the CIP participant group is
4 specific to slow brushing speeds and no difference was observed for faster speeds (10 cm s^{-1}
5 and 30 cm s^{-1}) (Fig. 1B and Supplementary Fig. 1). This suggests mutations in Nav1.7 have an
6 impact on the affective perception of gentle touch.

7 An extended examination, that included facial electromyography (EMG) as an indicator
8 of emotional responses, was conducted on three of the CIP participants (participant nos. 2, 3,
9 and 4) and in eight control participants (Fig. 2A). Again, consistent with Fig. 1B and previous
10 studies,^{4,7,33,34,40} control participants rated slow stroking touch as significantly more pleasant
11 and less intense than fast stroking touch (Fig. 2B). Facial EMG in healthy control participants
12 revealed that the pleasant slow stroking was associated with a relaxation of the corrugator
13 (frowning) facial muscle, whereas the less pleasant fast stroking was associated with a
14 contraction of the corrugator muscle (Fig. 2B).^{36,37,41} In contrast, the CIP participants showed no
15 consistent corrugator activity to slow or fast brushing (Fig. 2B-C). Despite not rating either
16 stimulus as more or less pleasant, the CIP participants were able to discriminate stimulus
17 intensity (Fig. 2B-C).

18 Thus, taken together, the *SCN9A* mutations influenced not only the perception of touch
19 pleasantness but also the emotional reactions to touch, as measured using facial EMG. As
20 expected, the *SCN9A* mutations did not influence basic sensory-discriminative perception of
21 brush stimuli.

22 **C-LTMRs express Nav1.7**

23 Nav1.7 is highly expressed in the peripheral nervous system with restricted expression within
24 the CNS in sub-cortical structures, including the thalamus, medial amygdala, hypothalamus, and
25 the axons of the olfactory epithelium projecting to the olfactory bulb.^{42,43} Nav1.7 is highly
26 expressed in C-nociceptors.⁴⁴ It has been shown through mRNA sequencing of the C-LTMR
27 population that they also express Nav1.7.⁴⁵ Using the data set provided by Reynders *et al*

1 (2015) we identified *SCN9A* expression in three sensory neuron populations: C-LTMRs
2 (GINIP+/IB4-), non-peptidergic nociceptors (GINIP+/IB4+) and all other DRG neurons (IB4-
3 /GINIP-), with the highest reads per kilobase of transcript per million mapped reads (RPKM)
4 seen in C-LTMRs (Supplementary Fig. 2). To validate these sequencing results, we carried out in
5 situ hybridisation (ISH) combined with immunohistochemistry (IHC) and Nav1.7mRNA was
6 indeed present in the C-LTMR population (GINIP+/IB4-) (Supplementary Fig. 2). We also found
7 that 99.4% of peptidergic nociceptors and 82.6% of myelinated fibres express Nav1.7mRNA
8 (Supplementary Fig. 2). More recently, Zheng *et al* (2019) carried out deep sequencing of seven
9 transgenically labelled sensory neuron populations, including C-LTMRs.¹⁷ This work
10 demonstrated that C-LTMRs identified by expression of TH, express Nav1.7mRNA and
11 surprisingly this expression is highest in C-LTMRs as compared to seven other sensory neuron
12 populations, including nociceptors.¹⁷ To validate these sequencing results from Zheng *et al*
13 (2019) and ask what proportion of C-LTMRs express Nav1.7, we carried out ISH and IHC and
14 found that 100% of TH positive C-LTMRs express Nav1.7 mRNA (Fig. 3A-B).

15 **The TH^{CreERT2} transgenic mouse efficiently targets C-LTMRs that express Nav1.7**

16 We characterised the TH^{CreERT2} line generated by Abaira *et al* (2017) as a means to target the C-
17 LTMR population. This line was previously used to demonstrate C-LTMR central projections
18 terminating in lamina III of the DH.⁴⁶ We bred the TH^{CreERT2} line with a Cre dependent tdTomato
19 reporter line and induced tdTomato expression in adulthood in order to characterise the C-
20 LTMR population at the level of the DRG, spinal cord and skin. We show that the labelled C-
21 LTMR population makes up $4.7 \pm 0.5\%$ of lumbar and $9.8 \pm 1.9\%$ of thoracic DRG neurons (Fig.
22 3C), similar to previous studies.¹³ We confirm that these neurons are indeed small neurons with
23 an average neuronal area of $238.6 \pm 8.3\mu\text{m}^2$ (an area below $490\mu\text{m}^2$ denotes a small DRG
24 neuron with a diameter of $25\mu\text{m}$). We have shown that the TH^{CreERT2} line is ~80% efficient at
25 targeting the population when tamoxifen is given in adulthood (Fig. 3C and Supplementary Fig.
26 3). The transgenically labelled TH positive C-LTMRs form a distinct non-overlapping population
27 that do not express/bind, Parvalbumin (PV), IB4, CGRP, or S100 β and all C-LTMRs express the
28 VGSC Nav1.8 (Fig. 3D-I, Supplementary Fig. 3).^{13,17} We have also shown consistent and expected

1 lamina III tdTomato C-LTMR terminations in the DH of the spinal cord (Supplementary Fig. 3) as
2 reported previously.⁴⁶ We assessed labelled sensory ending structures in the skin and identified
3 longitudinal lanceolate endings associated with hair follicles on hind paw hairy skin (Fig. 3J). It
4 has been recently reported that some species of rodent (including C57BL/6 strains) have hair
5 follicles located between the running pads of their paws, which have been evolutionary
6 conserved.⁴⁷ Interestingly, we found that C-LTMR sensory endings are also present on the
7 plantar surface of rodent glabrous skin and indeed innervate hairs located between rodent
8 running pads (Fig. 3K). While further evidence is needed in humans, a recent microneurography
9 study identified a small number of mechanosensitive units on glabrous skin with delayed
10 responses to mechanical stimulation suggesting that they may have C-LTMR characteristics.⁴⁸
11 Finally, we do not see any labelled epidermal small fibres (nociceptors) (Fig. 3L). Taken
12 together, all C-LTMRs express Nav1.7 mRNA and the TH^{CreERT2} line first published by Abaira *et al*
13 (2017) is a good model to study C-LTMR function.

14 **Genetic loss of Nav1.7 in rodent and human C-LTMRs results in mechanical and** 15 **cooling deficits**

16 To understand the role of Nav1.7 in mouse C-LTMRs we generated a conditional C-LTMR
17 specific Nav1.7 knock-out (KO) mouse, using the previously discussed TH^{CreERT2} model crossed
18 with a floxed Nav1.7 mouse line. Following administration of tamoxifen and the conditional KO
19 of Nav1.7 we conducted an array of behaviour assays in order to profile sensory function. We
20 found that mice lacking Nav1.7 in C-LTMRs exhibit a small but significant hyposensitivity to
21 punctate mechanical stimuli (Fig. 4A). This finding led to re-examination of CIP participant 4 and
22 their ability to discriminate between low force punctate monofilaments. Consistently, the
23 ability to discriminate between low force monofilaments was reduced in CIP participant 4
24 compared to 20 healthy control participants (Supplementary Fig. 4A). In addition, single-unit
25 microneurography recordings in healthy participants demonstrate that human C-LTMRs can
26 encode low force punctate stimuli (Supplementary Fig. 4B).

27 In our conditional KO mouse there was no difference in the number of responses to light
28 brush stimuli as measured by a cotton swab, brush or sticky tape (Fig. 4B-C, Supplementary Fig.

1 5A). In addition, loss of Nav1.7 specifically in C-LTMRs did not affect tactile acuity tasks
2 (Supplementary Fig. 5B-E). As expected, the loss of Nav1.7 in C-LTMRs did not affect the latency
3 to withdraw from a nociceptive hotplate (Fig. 4D). Mouse and human C-LTMRs are known to
4 respond to cooling stimuli and we have previously found that CIP participants show
5 hyposensitivity to cold and cool stimuli (Supplementary Fig. 4C). We therefore sought to assess
6 the loss of Nav1.7 in cold stimuli coding. We allowed mice to freely explore a temperature
7 gradient apparatus which ranged from 6-54°C for 30 minutes. Nav1.7-WT mice had a bell
8 shaped (inverted U-shaped) response to the thermal gradient apparatus while TH^{CreERT2}:Nav1.7-
9 KO mice spend more time in cooler zones as seen by a significant leftward shift in the non-
10 linear regression Gaussian curve (Fig. 4E), suggestive of a deficit in cool detection. Additionally,
11 there is a ~5°C reduction in the average preferred temperature (the temperature at which mice
12 spent most of their time) in TH^{CreERT2}:Nav1.7-KO mice (23.16 ± 1.59°C) compared to wildtype
13 mice (28.45 ± 1.21°C) (Fig. 4F). We also analysed our behavioural data in a sex-dependent
14 manner as it has been previously reported that mechanisms underlying thermal preference can
15 differ between male and female mice⁴⁹. The mechanical and thermal phenotypes were present
16 and consistent in both male and female mice (Supplementary Fig. 6A-G). Therefore, we
17 conclude that the behavioural consequence of Nav1.7 loss of function in C-LTMRs is not
18 sexually dimorphic. These data showing that the genetic loss of Nav1.7 in rodent and human C-
19 LTMRs results in mechanical and cool sensory deficits, illustrate that Nav1.7 is necessary for
20 normal C-LTMR function.

21 **C-LTMRs lacking Nav1.7 are hypo-excitabile**

22 Given the changes in sensory behaviour in the TH^{CreERT2}:Nav1.7-KO mice we wanted to confirm
23 our C-LTMR Nav1.7 KO and interrogate the contribution of Nav1.7 to the total sodium currents
24 in this population. We labelled the C-LTMR population using the Cre recombinase dependent
25 virus, AAV9.Flex.eGFP, giving us the ability to study C-LTMRs *in vitro*. We performed intrathecal
26 (i.t) injections of the reporter virus into TH^{CreERT2} and TH^{CreERT2}:Nav1.7^{fllox/fllox} mice and
27 administered tamoxifen one week later to initiate simultaneous eGFP expression and Nav1.7
28 ablation (Fig. 5A). Four weeks following tamoxifen dosing we cultured lumbar DRG neurons

1 from injected animals and performed voltage clamp analysis and single cell qPCR on eGFP
2 positive cells (Fig. 5B). Using single cell qPCR, we confirm that the Nav1.7 mRNA transcript level
3 is significantly reduced in TH^{CreERT2}:Nav1.7-KO C-LTMRs (Supplementary Fig. 7). Voltage clamp
4 recordings showed a reduced peak inward current upon membrane depolarisation to 0mV (Fig.
5 5C-D) and lower current densities across a range of voltages at which voltage-gated sodium
6 channels are known to activate (Fig. 5E-F), in C-LTMRs that lack Nav1.7 compared to wild type
7 neurons.

8 Due to Nav1.7's large contribution to sodium currents in C-LTMRs, we next examined
9 whether Nav1.7 directly regulated C-LTMR excitability. We performed single-fibre primary
10 afferent characterisation of C-LMTRs in hind paw hairy skin from Nav1.7-WT and
11 TH^{CreERT2}:Nav1.7-KO mice. The conduction velocity of recorded C-LTMRs were within the mouse
12 C-fibre range (<1.2m/s) and comparable between both WT and KO mice (Fig. 5G). However,
13 there was a significant increase in the mechanical thresholds of TH^{CreERT2}:Nav1.7-KO C-LTMRs
14 compared to those recorded from Nav1.7-WT mice (Fig. 5H). We next analysed the stimulus
15 response functions of C-LTMRs to suprathreshold punctate mechanical stimuli. and saw that C-
16 LTMRs lacking Nav1.7 fire less and display significant hypo-excitability (Fig. 5I-J). We also
17 investigated the stimulus response functions in response to repeated punctate mechanical
18 stimuli, where each stimulus (which is a downward indentation of the skin) increases its
19 velocity. Rodent C-LTMRs from TH^{CreERT2}:Nav1.7-KO mice exhibited a reduced firing frequency
20 and are hypo-excitabile to moving punctate stimuli compared to Nav1.7-WT mice (Fig. 5K).

21 Due to the mouse behavioural and human psychophysical data demonstrating cool
22 detection abnormalities, we directly analysed the temperature sensibility of mouse C-LTMRs
23 (Fig. 5L-N). Using three temperature ramps restricted to identified C-LTMR receptive fields,
24 their response to cooling and warming stimuli was assessed in detail. The first temperature
25 ramp started at 31°C (skin temperature) and cooled the receptive field to 14°C (Fig. 5L), the
26 second ramp warmed the receptive field from 14°C to 42°C (Fig. 5M), and finally the third ramp
27 cooled the receptive field from 42°C to 14°C (Fig. 5N). Our data demonstrates that wildtype C-
28 LTMRs respond to both cooling and warming stimuli with a stimulus response that resembles

1 an inverted U-shaped response with a maximal firing at $\sim 27-28^{\circ}\text{C}$. However, C-LTMRs lacking
2 functional Nav1.7 responded less and exhibited hypo-excitability to cooling stimuli (Fig. 5L-N).
3 Collectively, we demonstrate that Nav1.7 is a key regulator of C-LTMR excitability in response
4 to mechanical and cool stimuli.

5 **Small molecule blockade of Nav1.7 reduces C-LTMR excitability**

6 The small molecule inhibitor PF-05089771 shows selectivity for Nav1.7³⁹ and has been in clinical
7 development; it has shown potential in the treatment of patients with inherited
8 erythromelalgia.³⁰ We performed voltage clamp recordings of labelled C-LTMRs (Fig. 6A) to
9 investigate the effects of PF-05089771 (10nM) on sodium current densities. Small molecule
10 blockade of Nav1.7 significantly reduced the total sodium current density in C-LTMRs (Fig. 6B-
11 C). We next performed primary afferent recordings using the *ex vivo* skin-nerve preparation
12 from wildtype mice in the presence or absence of PF-05089771, to determine whether this
13 selective small molecule blocker could impact on rodent C-LTMR function. We identified and
14 isolated C-LTMR receptive fields in rodent hairy skin and applied either vehicle or PF-05089771
15 and assessed excitability (Fig. 6D). We compared the mechanical thresholds of the isolated C-
16 LTMR receptive fields before and after treatment and discovered that, compared to vehicle,
17 there is a significant increase in mechanical thresholds when PF-05089771 was applied (Fig. 6E).
18 In addition, when we assessed the stimulus response function using suprathreshold mechanical
19 stimuli we observed a significant reduction in C-LTMR activity in the presence of PF-05089771
20 compared to vehicle (Fig. 6F). To summarise, selective small molecule blockade of Nav1.7
21 reduced total sodium currents and altered C-LTMR function, resulting in hypo-excitability.

22 **Computational modelling of human *SCN9A* mutations in C-LTMRs**

23 Given the clinical data that CIP participants experience an altered affective touch perception
24 and that genetic ablation or pharmacological blockade of Nav1.7 in the rodents reduced C-
25 LTMR excitability, we investigated the impact of the CIP participant mutations on C-LTMR
26 excitability and function. To address this question we took advantage of the recent Nav1.7
27 mutation characterisation from three CIP participants in our cohort.²⁶ From the data available

1 in McDermott *et al* (2019) we were able to calculate the fold decrease in Nav1.7 conductance
2 as a consequence of each *SCN9A* mutation (Supplementary Table 2). We used a publicly
3 available computational model of C-LTMR sensory neurons¹⁷ to model C-LTMR excitability while
4 altering the Nav1.7 conductance (Nav1.7 g-CLTMR, mS/cm²) in accordance with the
5 conductance decrease observed in each CIP participant mutation (Supplementary Table 2). We
6 ran the model in the naïve setting, without changing the Nav1.7 conductance, in order to
7 resemble healthy control participant excitability measures (Fig. 7A). Next, we ran the model for
8 four mutations, from three CIP participants using the new Nav1.7 conductance values
9 calculated (Fig. 7B-E). C-LTMR excitability is strikingly impaired when modelling CIP participant
10 mutations in C-LTMRs (Fig. 7B-E). The minimum current required to elicit an action potential in
11 healthy control models is 40pA; however, in CIP mutation models this ranges from 550pA-
12 767pA, depending on the mutation (Fig. 7A-E). We also used these computational models to
13 look at suprathreshold excitability. We modelled C-LTMRs receiving incremental current
14 injections ($\Delta 25\text{pA}$) and there is clear hypo-excitability observed in all CIP mutation models
15 compared to the healthy control model (Fig. 7F-K).

16 Discussion

17 We have found that not only do humans with *SCN9A*-LOF mutations have CIP but also an
18 altered experience and perception of affective touch sensation. We demonstrate that rodent C-
19 LTMRs express high levels of Nav1.7. We used selective genetic strategies in order to attribute
20 this LOF phenotype to hypo-excitability C-LTMR primary afferents. We also challenged the
21 current concept that pharmacological/genetic blockade of Nav1.7 is selective to the nociceptive
22 system and show the affective touch system, cool sensitivity, and mechanical low force
23 discrimination also requires functional Nav1.7.

24 The CIP participants in this study were all bi-allelic compound heterozygotes and all of
25 their mutations have been previously characterised and shown to abolish almost all Nav1.7
26 driven sodium currents. The CIP participants have also been extensively studied in the context
27 of nociception.^{18,24} These participants have never experienced pain and as a result have had
28 multiple injuries throughout their lives due to a loss of functional C-nociceptors, a lack of

1 epidermal small fibres²⁶ and up-regulation of endogenous opioids.²⁸ These patients also lack
2 itch perception in response to pruritogens, such as histamine, and demonstrate mild
3 hyposensitivity to warm and cool stimuli which we have re-examined in this study. Vibration
4 and mechanical detection thresholds have not been found to differ from control individuals,
5 however we also re-examined low force punctate monofilament discrimination in this study
6 using a sensitive measure of light touch discrimination. The affective touch system has never
7 previously been investigated in these patients.

8 The identification of human mutations affecting C-LTMRs and affective touch is scarce.
9 Patients with hereditary sensory and autonomic neuropathy type-III (HSAN-III) and type-V
10 (HSAN-V) who also have congenital insensitivity to pain have a reduced affective touch
11 percept.^{50,51} HSAN-V is due to a mutation in the nerve growth factor beta (*NGFβ*) gene NGF
12 mediates its effects by binding to the NTRK1 receptor; the NTRK1⁺ lineage of small diameter,
13 unmyelinated neurons derive from the *Neurog1* wave of neurogenesis and these mutations
14 likely impede the initial development of C-LTMRs.⁵² We have now shown that LOF mutations in
15 the voltage-gated ion channel Nav1.7, have negative impacts on affective touch. Our cohort of
16 CIP participants exhibit altered self-reported pleasantness for gentle dynamic brush stimulation
17 and do not show the classical inverted U-shaped response pattern. Compared to a less pleasant
18 fast touch stimulus, affective touch reliably attenuates activity of the corrugator muscle in an
19 unbiased facial EMG measure of affective touch, suggestive of a reduction in negative
20 affect.^{36,41} Here, 3 cm s⁻¹ stroking stimulation failed to influence corrugator activity in CIP
21 participants, suggesting that touch which normally elicits human C-LTMR activation does not
22 attenuate negative affect in these individuals. This was selective for the affective aspect of the
23 stimulus, since all CIP participants were able to rate the stimulus intensity similarly to healthy
24 control participants. This confirms previous literature suggesting that in cases of CIP the large
25 myelinated touch fibres are not compromised.²⁶ Collectively, these findings demonstrate that
26 human *SCN9A*-LOF mutations can alter the affective component of pleasant touch sensation.

27 To interrogate further the expression and role of Nav1.7 in C-LTMR function we took
28 advantage of rodent models. Nav1.7 is known to be expressed in nociceptors.^{44,53} Using ISH and

1 analysis of previously published data sets⁴⁵ we show that *SCN9A* is expressed in all C-LTMRs and
2 to a high level. This data adds to the recent sequencing data showing that *SCN9A* is most highly
3 expressed in the rodent C-LTMR population.¹⁷ Interestingly many A-LTMRs also show some
4 expression of Nav1.7 however there is no evidence of impairments either in large fibre
5 mediated touch modalities or the electrophysiological properties of these afferents in *SCN9A*-
6 LOF CIP patients.²⁴⁻²⁶ This is likely due to functional redundancy and the co-expression (unlike in
7 C-LTMRs¹⁷) of other TTX-S VGSCs such as Nav 1.1 and 1.6 in these neurons which can
8 compensate for the loss of Nav1.7.¹⁸

9 A number of genetic knock-out strategies have previously been used to investigate the
10 role of C-LTMRs in rodents. A global vGLUT3 KO, initially thought to be C-LTMR specific, resulted
11 in altered noxious mechanical thresholds,¹⁰ a phenotype which was later shown to be driven by
12 loss of spinal vGLUT3.⁵⁴ Other studies suggest deficits in acute light touch, cold detection and
13 chemical pain responses when Nav1.8 positive sensory neurons (which include C-LTMRs) lack
14 Cav3.2 a voltage-gated calcium channel enriched in C-LTMRs.⁵⁵ In contrast, the global KO of the
15 chemokine-like protein Tafa4, which is thought to only be expressed and released by C-LTMRs,
16 resulted in a pro-nociceptive phenotype.¹¹ This phenotype was recovered by administration of
17 exogenous Tafa4 a mechanism which involves GABAergic transmission and spinal microglia.^{11,56}
18 Vrontou *et al.* (2013) identified a population of sensory neurons that expressed the G-protein
19 coupled receptor MrgprB4 and provided evidence that this population is involved in massage-
20 like stroking of hairy skin.⁵⁷ Unfortunately, Vrontou *et al.* (2013) were not able to identify this
21 population as a low-threshold mechanoreceptive population and could only infer that they are
22 C-fibres due to molecular traits;^{57,58} there has been no physiological evidence of C-fibre range
23 conduction velocities of MrgprB4+ afferents. There is closer alliance and more physiological
24 evidence that the TH/vGLUT3/Tafa4 population is the likely species equivalent of human C-
25 LTMRs.^{10,11,13} However, we cannot exclude the possibility that both populations co-exist and
26 that perhaps relates to modality specific pleasure perception.

27 Hitherto, there has been a lack of transgenic tools available to selectively target the C-
28 LTMR population; the discovery that TH is a marker of C-LTMRs and development of the

1 TH^{CreERT2} line has helped delineate the physiology and connectivity of these neurons in the
2 rodent.^{13,46} In validating the TH^{CreERT2} mouse (and confirming that it is a good model system to
3 target C-LTMRs⁴⁶) we have found that C-LTMRs innervate not only hairy skin on the dorsum of
4 the paw but also the hind-paw plantar surface of mice, as longitudinal lanceolate endings.
5 These hair follicles located between hind-paw running pads were thought to exclusively be
6 innervated by the A δ -LTMR population known as D-hairs.^{47,59} This is an important finding, as
7 studies often overlook these particular hair follicles and the dogma currently suggests that C-
8 LTMRs do not innervate rodent plantar skin. We provide evidence that this population does
9 innervate the running pad region, which is commonly tested in rodent sensory biology.

10 We saw that when we ablated Nav1.7 in rodent C-LTMRs, the response to noxious heat
11 remained intact but mice became hyposensitive to punctate mechanical stimuli. This finding is
12 consistent with previous rodent studies which also show that C-LTMRs have a modest
13 contribution to punctate mechanical stimuli.⁵⁵ There is debate in the literature over the
14 perceptual correlate of a withdrawal to a von Frey hair in rodents. It is likely that von Frey
15 withdrawal relates to stimulus detection rather than a painful aversion. The genetic ablation of
16 all TRPV1-lineage neurons (all nociceptors) or the optogenetic silencing of CGRP+ peptidergic
17 neurons did not alter von Frey thresholds in the naïve, uninjured state.^{60,61} In addition, the early
18 human data which first characterised C-LTMRs, and human microneurography we present in
19 this study demonstrates that C-LTMRs do respond to both punctate and brush stimuli.³
20 Furthermore, patients lacking A-fibre function are still able to detect low-force punctate
21 monofilaments, but only in hairy skin.⁶² This led us to reassess low-force punctate
22 monofilament discrimination in a single CIP participant using a more sensitive tactile task (more
23 sensitive than QST). We show for the first time that human C-LTMRs can encode low
24 indentation forces in healthy participants, and that the ability to discriminate between low
25 force punctate mechanical stimuli was reduced in one of the *SCN9A*-LOF participants. Together
26 this highlights that C-LTMRs play a role in punctate mechanical detection in rodents and
27 humans.

1 Interestingly, using our mouse KO model we did not see any changes in the number of
2 responses to dynamic light touch stimuli. However, one must consider that these assays
3 interrogate stimulus detection, not the affective component of the stimulus. Our human data
4 suggest that *SCN9A*-LOF participants do not fail to detect the brush stimuli, but that it is the
5 affective perception that is altered. The sensory biology field is currently challenged in not
6 having a reliable read-out for affective pleasure sensation in rodents, an obstacle that as a
7 community we need to overcome. The loss of C-LTMR Nav1.7 resulted in deficits in cool coding
8 at the behavioural and electrophysiological level (discussed further below), suggesting without
9 Nav1.7 in the C-LTMR population mice are unable to properly code thermal stimuli. One idea is
10 that C-LTMR activity contributes to thermal preference and loss of Nav1.7 leads to mice having
11 altered thermal preferences and spending a longer amount of time in cooler (non-noxious)
12 regions. We have re-examined human data from a previous study, quantitative sensory testing
13 (QST) was used to sensory profile CIP participants and it was found that they are hypo-sensitive
14 to cooling stimuli.²⁶ There are few other regions of the nervous system that also co-express TH
15 and Nav1.7 where ablation would also occur in our model. Sympathetic neurons express both¹⁵
16 however they unlikely require Nav1.7 as CIP participants do not present with sympathetic
17 deficits. Equally, dopaminergic neurons in the periaqueductal grey and ventral midbrain express
18 TH but show very low levels of *SCN9A* expression.¹⁵ Finally, some populations of jugular/nodose
19 sensory neurons express both TH and Nav1.7.⁶³ However, it is reported that TH+ jugular sensory
20 neurons can be molecularly classified as C-LTMRs⁶³, and nodose sensory neurons innervate
21 visceral organs, i.e. not regions we have tested in this study.

22 Given the high expression of Nav1.7 in C-LTMRs and the important role of Nav1.7 as a
23 threshold channel within sensory neurons, we investigated whether the observed behavioural
24 changes were due to alterations in the excitability of C-LTMRs. Using voltage-clamp recordings
25 *in vitro*, we saw a reduction of the sodium current density in C-LTMRs which lack Nav1.7 and
26 found that there is a large contribution of Nav1.7 to the total sodium currents in this
27 population. A previous study used the skin-nerve preparation to interrogate Nav1.7
28 contribution to peripheral nerve excitability, using a sensory neuron specific Nav1.7 KO
29 mouse.⁶⁴ However, Hoffmann *et al.* (2018), only recorded from two C-LTMRs units in each

1 genotype and were therefore underpowered to draw any conclusions as to the stimulus-
2 response function of these units. We therefore assessed C-LTMR primary afferent terminal
3 excitability in detail using the skin-nerve preparation. Here, we found that loss of Nav1.7 results
4 in hypo-excitability and, in particular, alters C-LTMR stimulus response functions to static and
5 dynamic punctate mechanical stimuli. In addition, we directly characterised the thermal
6 response of C-LTMRs and observed that they respond to both warming and cooling stimuli with
7 an activity pattern that is consistent with an inverted U-shaped response. Peak activity of this
8 inverted U-shaped response was $\sim 27-28^{\circ}\text{C}$ which is consistent with our thermal gradient
9 behavioural finds where thermal preference is also $\sim 28^{\circ}\text{C}$ in wildtype mice. The relationship
10 between thermal response of C-LTMRs and subjective preference in mice resonates well with
11 human evidence. In a combined microneurography and psychophysics experiment, Ackerley *et*
12 *al.* (2014) replicated the typical speed-dependent inverted U-shaped response in C-LTMRs,
13 together with increased C-LTMRs firing to neutral (32°C) compared to warm (42°C) and cool
14 (18°C) stroking temperatures.⁶⁵ Importantly, the speed-dependent vigorous response to
15 stroking stimuli at neutral temperatures was positively correlated to self-reported pleasantness,
16 indicating a link between C-LTMRs firing properties and subjective preference. Our findings in
17 the mouse also support previous studies that implicate C-LMTRs in thermal preference. TH
18 positive C-LTMRs are sensitive to cooling stimulation¹³ and an altered thermal preference was
19 observed in mice with hyposensitive C-LTMRs due to lack of the voltage-gated calcium channel
20 Cav3.2.⁵⁵ Additionally, recent work used activatory chemogenetic tools to selectively increase
21 C-LTMR activity, which resulted in increased thermal preference and induced a place
22 preference in mice.⁶⁶ We provide evidence that C-LTMR activity in response to thermal stimuli
23 may underlie thermal preference in mice. These findings are consistent with the idea that C-
24 LTMR activity may underlie social thermoregulation, such as mammalian huddling behaviours,
25 which are important for survival.⁶⁷ Thermal characterisation following Nav1.7 ablation in C-
26 LTMRs results in hypo-excitability of C-LTMRs to cooling stimuli, a loss of the inverted U-shaped
27 response pattern and a shift in the thermal preference of behaving mice.

28 From this, we propose a mechanism whereby Nav1.7, which has a large contribution to
29 C-LTMR sodium currents, is important in regulating C-LTMR excitability and function. We

1 propose that loss of functional Nav1.7 in our CIP participants likely results in hypo-excitability C-
2 LTMRs which can no longer effectively drive the affective component of pleasant brush stimuli,
3 monofilament discrimination and cool sensibility.

4 Targeting Nav1.7 to therapeutically treat painful conditions may therefore have
5 unintended consequences on C-LTMRs and the affective touch system. We indeed saw a
6 reduction in both C-LTMR sodium currents and terminal excitability when using a Nav1.7
7 selective small molecule blocker. These data suggest that current and future strategies, which
8 target Nav1.7 to treat pain, need to consider the consequences of reducing the excitability, of
9 this non-nociceptive population and how this might, alter social touch, relationships and
10 regulation of stress response.⁶⁷ Whether these are clinically relevant side effects remains
11 unknown.

12 We know from previous studies that human *SCN9A*-LOF mutations can reduce neuronal
13 excitability.^{26,29} As such, we sought to investigate the effects of human *SCN9A*-LOF mutations in
14 the context of C-LTMRs, using a recently developed C-LTMR computational model.¹⁷ While
15 models of C-nociceptors exist there is strong evidence that these would not generalise to C-
16 LTMRs.⁶⁸ For instance, C-LTMR activity dependent slowing is very different compared to C-
17 nociceptors.^{69,70} Therefore, using a C-LTMR specific computational model, we recapitulated
18 mutations from a subset of our CIP participant cohort and consistent with our empirical findings
19 these led to hypo-excitability in C-LTMRs. The CIP-participants have compound heterozygote
20 mutations so the outcome *in vivo* is the combinatorial effect of two mutations.

21 To summarise, we have used a multidisciplinary approach to investigate the role of
22 Nav1.7 in C-LTMR function in humans and mice. Psychophysical testing showed CIP participants
23 have an altered perception of affective touch sensation, deficits in low-force monofilament
24 discrimination and cool sensibility. We used a mouse model to selectively ablate Nav1.7 in C-
25 LTMRs in order to determine this mechanism. We found that loss of Nav1.7 in C-LTMRs results
26 in behavioural hyposensitivity to punctate mechanical stimuli, deficits in cool sensibility and an
27 altered thermal preference. Loss of Nav1.7 in C-LTMRs results in a reduction in sodium currents
28 and hypo-excitability to mechanical and cooling stimuli. Pharmacological blockade of Nav1.7

1 also led to hypo-excitability C-LTMRs. The impact of loss of function in one VGSC alpha subunit
2 within different sensory neuron sub-populations is dependent on co-expression with other
3 VGSCs, which vary between fibre types, and the non-redundant role of Nav1.7 in C-LTMRs
4 which we observed, is supported by a recent computational model of C-LTMRs. The phenotype
5 of bi-allelic LOF gene mutations in *SCN9A* has therefore been widened to not only include pain
6 perception but also impaired pleasant touch perception. Furthermore, targeting Nav1.7 to
7 therapeutically treat painful conditions may have implications on the affective touch system.

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22 **Competing interests**

23 DLB has acted as a consultant on behalf of Oxford Innovation for Abide, Amgen, G Mitsubishi
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25 **Supplementary material**

26 Supplementary material is available at *Brain* online.

1 **References**

- 2 1. Zotterman Y. Touch, pain and tickling: an electro-physiological investigation on
3 cutaneous sensory nerves. *J Physiol.* 1939;95(1):1-28.
4 doi:10.1113/jphysiol.1939.sp003707
- 5 2. Nordin M. Low-threshold mechanoreceptive and nociceptive units with unmyelinated (C)
6 fibres in the human supraorbital nerve. *J Physiol.* 1990;426:229-240.
7 doi:10.1113/jphysiol.1990.sp018135
- 8 3. Vallbo ÅB, Olausson H, Wessberg J. Unmyelinated Afferents Constitute a Second System
9 Coding Tactile Stimuli of the Human Hairy Skin. *J Neurophysiol.* 1999;81(6):2753-2763.
10 doi:10.1152/jn.1999.81.6.2753
- 11 4. Morrison I, Björnsdotter M, Olausson H. Vicarious responses to social touch in posterior
12 insular cortex are tuned to pleasant caressing speeds. *J Neurosci.* 2011;31(26):9554-
13 9562. doi:10.1523/JNEUROSCI.0397-11.2011
- 14 5. Olausson H, Wessberg J, Morrison I, McGlone F, Vallbo Å. The neurophysiology of
15 unmyelinated tactile afferents. *Neurosci Biobehav Rev.* 2010;34(2):185-191.
16 doi:10.1016/J.NEUBIOREV.2008.09.011
- 17 6. Liljencrantz J, Strigo I, Ellingsen DM, et al. Slow brushing reduces heat pain in humans.
18 *Eur J Pain.* 2017;21(7):1173-1185. doi:10.1002/ejp.1018
- 19 7. Löken LS, Wessberg J, Morrison I, McGlone F, Olausson H. Coding of pleasant touch by
20 unmyelinated afferents in humans. *Nat Neurosci.* 2009;12(5):547-548.
21 doi:10.1038/nn.2312
- 22 8. Lynn B, Carpenter SE. Primary afferent units from the hairy skin of the rat hind limb.
23 *Brain Res.* 1982;238(1):29-43. doi:10.1016/0006-8993(82)90768-5
- 24 9. Leem JW, Willis WD, Chung JM. Cutaneous sensory receptors in the rat foot.
25 <https://doi.org/101152/jn19936951684>. 1993. doi:10.1152/JN.1993.69.5.1684

- 1 10. Seal RP, Wang X, Guan Y, et al. Injury-induced mechanical hypersensitivity requires C-low
2 threshold mechanoreceptors. *Nature*. 2009;462(7273):651-655.
3 doi:10.1038/nature08505
- 4 11. Delfini M-C, Mantilleri A, Gaillard S, et al. TFAA4, a Chemokine-like Protein, Modulates
5 Injury-Induced Mechanical and Chemical Pain Hypersensitivity in Mice. *Cell Rep*.
6 2013;5(2):378-388. doi:10.1016/J.CELREP.2013.09.013
- 7 12. Urien L, Gaillard S, Lo Re L, et al. Genetic ablation of GINIP-expressing primary sensory
8 neurons strongly impairs Formalin-evoked pain. *Sci Rep*. 2017;7(1):43493.
9 doi:10.1038/srep43493
- 10 13. Li L, Rutlin M, Abraira VE, et al. The Functional Organization of Cutaneous Low-Threshold
11 Mechanosensory Neurons. *Cell*. 2011;147(7):1615-1627. doi:10.1016/J.CELL.2011.11.027
- 12 14. Usoskin D, Furlan A, Islam S, et al. Unbiased classification of sensory neuron types by
13 large-scale single-cell RNA sequencing. *Nat Neurosci*. 2015;18(1):145-153.
14 doi:10.1038/nn.3881
- 15 15. Zeisel A, Hochgerner H, Lönnerberg P, et al. Molecular Architecture of the Mouse
16 Nervous System. *Cell*. 2018;174(4):999-1014.e22. doi:10.1016/J.CELL.2018.06.021
- 17 16. Larsson M, Broman J. Synaptic Organization of VGLUT3 Expressing Low-Threshold
18 Mechanosensitive C Fiber Terminals in the Rodent Spinal Cord. *eNeuro*.
19 2019;6(1):ENEURO.0007-19.2019. doi:10.1523/ENEURO.0007-19.2019
- 20 17. Zheng Y, Liu P, Bai L, Trimmer JS, Bean BP, Ginty DD. Deep Sequencing of Somatosensory
21 Neurons Reveals Molecular Determinants of Intrinsic Physiological Properties. *Neuron*.
22 June 2019. doi:10.1016/J.NEURON.2019.05.039
- 23 18. Bennett DL, Clark AJ, Huang J, Waxman SG, Dib-Hajj SD. The Role of Voltage-Gated
24 Sodium Channels in Pain Signaling. *Physiol Rev*. 2019;99(2):1079-1151.
25 doi:10.1152/physrev.00052.2017

- 1 19. Yang Y. Mutations in SCN9A, encoding a sodium channel alpha subunit, in patients with
2 primary erythralgia. *J Med Genet*. 2004;41(3):171-174. doi:10.1136/jmg.2003.012153
- 3 20. Dib-Hajj SD, Rush AM, Cummins TR, et al. Gain-of-function mutation in Nav1.7 in familial
4 erythromelalgia induces bursting of sensory neurons. *Brain*. 2005;128(8):1847-1854.
5 doi:10.1093/brain/awh514
- 6 21. Fertleman CR, Baker MD, Parker KA, et al. SCN9A Mutations in Paroxysmal Extreme Pain
7 Disorder: Allelic Variants Underlie Distinct Channel Defects and Phenotypes. *Neuron*.
8 2006;52(5):767-774. doi:10.1016/J.NEURON.2006.10.006
- 9 22. Faber CG, Hoeijmakers JGJ, Ahn H-S, et al. Gain of function Nav1.7 mutations in
10 idiopathic small fiber neuropathy. *Ann Neurol*. 2012;71(1):26-39. doi:10.1002/ana.22485
- 11 23. Blesneac I, Themistocleous AC, Fratter C, et al. Rare Nav1.7 variants associated with
12 painful diabetic peripheral neuropathy. *Pain*. 2018;159(3):469.
13 doi:10.1097/J.PAIN.0000000000001116
- 14 24. Cox JJ, Reimann F, Nicholas AK, et al. An SCN9A channelopathy causes congenital inability
15 to experience pain. *Nature*. 2006;444(7121):894-898. doi:10.1038/nature05413
- 16 25. Goldberg Y, MacFarlane J, MacDonald M, et al. Loss-of-function mutations in the Nav1.7
17 gene underlie congenital indifference to pain in multiple human populations. *Clin Genet*.
18 2007;71(4):311-319. doi:10.1111/j.1399-0004.2007.00790.x
- 19 26. McDermott LA, Weir GA, Themistocleous AC, et al. Defining the Functional Role of
20 Nav1.7 in Human Nociception. *Neuron*. 2019;101(5):905-919.e8.
21 doi:10.1016/J.NEURON.2019.01.047
- 22 27. Weiss J, Pyrski M, Jacobi E, et al. Loss-of-function mutations in sodium channel Nav1.7
23 cause anosmia. *Nature*. 2011;472(7342):186-190. doi:10.1038/nature09975
- 24 28. Minett MS, Pereira V, Sikandar S, et al. Endogenous opioids contribute to insensitivity to
25 pain in humans and mice lacking sodium channel Nav1.7. *Nat Commun*. 2015;6(1):8967.

- 1 doi:10.1038/ncomms9967
- 2 29. Shields SD, Deng L, Reese RM, et al. Insensitivity to Pain upon Adult-Onset Deletion of
3 Nav1.7 or Its Blockade with Selective Inhibitors. *J Neurosci*. 2018;38(47):10180-10201.
4 doi:10.1523/JNEUROSCI.1049-18.2018
- 5 30. Cao L, McDonnell A, Nitzsche A, et al. Pharmacological reversal of a pain phenotype in
6 iPSC-derived sensory neurons and patients with inherited erythromelalgia. *Sci Transl
7 Med*. 2016;8(335):335ra56. doi:10.1126/scitranslmed.aad7653
- 8 31. Meents JE, Bressan E, Sontag S, et al. The role of Nav1.7 in human nociceptors: insights
9 from human induced pluripotent stem cell-derived sensory neurons of erythromelalgia
10 patients. *Pain*. 2019;160(6):1327-1341. doi:10.1097/j.pain.0000000000001511
- 11 32. Mis MA, Yang Y, Tanaka BS, et al. Resilience to Pain: A Peripheral Component Identified
12 Using Induced Pluripotent Stem Cells and Dynamic Clamp. *J Neurosci*. 2019;39(3):382-
13 392. doi:10.1523/JNEUROSCI.2433-18.2018
- 14 33. Perini I, Morrison I, Olausson H. Seeking pleasant touch: neural correlates of behavioral
15 preferences for skin stroking. *Front Behav Neurosci*. 2015;9:8.
16 doi:10.3389/fnbeh.2015.00008
- 17 34. Sehlstedt I, Ignell H, Backlund Wasling H, Ackerley R, Olausson H, Croy I. Gentle touch
18 perception across the lifespan. *Psychol Aging*. 2016;31(2):176-184.
19 doi:10.1037/pag0000074
- 20 35. Fridlund AJ, Cacioppo JT. Guidelines for Human Electromyographic Research.
21 *Psychophysiology*. 1986;23(5):567-589. doi:10.1111/j.1469-8986.1986.tb00676.x
- 22 36. Mayo LM, Lindé J, Olausson H, Heilig M, Morrison I. Putting a good face on touch: Facial
23 expression reflects the affective valence of caress-like touch across modalities. *Biol
24 Psychol*. 2018;137:83-90. doi:10.1016/J.BIOPSYCHO.2018.07.001
- 25 37. Ree A, Morrison I, Olausson H, Sailer U, Heilig M, Mayo LM. Using Facial

- 1 Electromyography to Assess Facial Muscle Reactions to Experienced and Observed
2 Affective Touch in Humans. *J Vis Exp*. 2019;(145):e59228. doi:10.3791/59228
- 3 38. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving Bioscience
4 Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol*.
5 2010;8(6):e1000412. doi:10.1371/journal.pbio.1000412
- 6 39. Alexandrou AJ, Brown AR, Chapman ML, et al. Subtype-Selective Small Molecule
7 Inhibitors Reveal a Fundamental Role for Nav1.7 in Nociceptor Electrogenesis, Axonal
8 Conduction and Presynaptic Release. Price TJ, ed. *PLoS One*. 2016;11(4):e0152405.
9 doi:10.1371/journal.pone.0152405
- 10 40. Triscoli C, Olausson H, Sailer U, Ignell H, Croy I. CT-optimized skin stroking delivered by
11 hand or robot is comparable. *Front Behav Neurosci*. 2013;7:208.
12 doi:10.3389/fnbeh.2013.00208
- 13 41. Ree A, Mayo LM, Leknes S, Sailer U. Touch targeting C-tactile afferent fibers has a unique
14 physiological pattern: A combined electrodermal and facial electromyography study. *Biol*
15 *Psychol*. 2019;140:55-63. doi:10.1016/J.BIOPSYCHO.2018.11.006
- 16 42. Kanellopoulos AH, Koenig J, Huang H, et al. Mapping protein interactions of sodium
17 channel Na_v 1.7 using epitope-tagged gene-targeted mice. *EMBO J*. 2018;37(3):427-445.
18 doi:10.15252/embj.201796692
- 19 43. Branco T, Tozer A, Magnus CJ, et al. Near-Perfect Synaptic Integration by Nav1.7 in
20 Hypothalamic Neurons Regulates Body Weight. *Cell*. 2016;165(7):1749-1761.
21 doi:10.1016/j.cell.2016.05.019
- 22 44. Black JA, Frézel N, Dib-Hajj SD, Waxman SG. Expression of Nav1.7 in DRG neurons
23 extends from peripheral terminals in the skin to central preterminal branches and
24 terminals in the dorsal horn. *Mol Pain*. 2012;8:82. doi:10.1186/1744-8069-8-82
- 25 45. Reynders A, Mantilleri A, Malapert P, et al. Transcriptional Profiling of Cutaneous
26 MRGPRD Free Nerve Endings and C-LTMRs. *Cell Rep*. 2015;10(6):1007-1019.

- 1 doi:10.1016/J.CELREP.2015.01.022
- 2 46. Abraira VE, Kuehn ED, Chirila AM, et al. The Cellular and Synaptic Architecture of the
3 Mechanosensory Dorsal Horn. *Cell*. 2017;168(1-2):295-310.e19.
4 doi:10.1016/J.CELL.2016.12.010
- 5 47. Walcher J, Ojeda-Alonso J, Haseleu J, et al. Specialized mechanoreceptor systems in
6 rodent glabrous skin. *J Physiol*. 2018;596(20):4995-5016. doi:10.1113/JP276608
- 7 48. RH W, M D, R A, H BW, J W, LS L. Evidence for sparse C-tactile afferent innervation of
8 glabrous human hand skin. *J Neurophysiol*. 2021;125(1):232-237.
9 doi:10.1152/JN.00587.2020
- 10 49. Bohic M, Ne Marics I, Santos C, Le Feuvre Y, Saurin AJ, Correspondence AM. Loss of
11 bhlha9 Impairs Thermotaxis and Formalin-Evoked Pain in a Sexually Dimorphic Manner.
12 2020. doi:10.1016/j.celrep.2019.12.041
- 13 50. Morrison I, Löken LS, Minde J, et al. Reduced C-afferent fibre density affects perceived
14 pleasantness and empathy for touch. *Brain*. 2011;134(4):1116-1126.
15 doi:10.1093/brain/awr011
- 16 51. Macefield VG, Norcliffe-Kaufmann L, Löken L, Axelrod FB, Kaufmann H. Disturbances in
17 affective touch in hereditary sensory & autonomic neuropathy type III. *Int J*
18 *Psychophysiol*. 2014;93(1):56-61. doi:10.1016/j.ijpsycho.2014.04.002
- 19 52. Marmigère F, Ernfors P. Specification and connectivity of neuronal subtypes in the
20 sensory lineage. *Nat Rev Neurosci*. 2007;8(2):114-127. doi:10.1038/nrn2057
- 21 53. Rostock C, Schrenk-Siemens K, Pohle J, Siemens J. Human vs. Mouse Nociceptors –
22 Similarities and Differences. *Neuroscience*. 2018;387:13-27.
23 doi:10.1016/j.neuroscience.2017.11.047
- 24 54. Peirs C, Williams S-PG, Zhao X, et al. Dorsal Horn Circuits for Persistent Mechanical Pain.
25 *Neuron*. 2015;87(4):797-812. doi:10.1016/J.NEURON.2015.07.029

- 1 55. François A, Schüetter N, Laffray S, et al. The Low-Threshold Calcium Channel Cav3.2
2 Determines Low-Threshold Mechanoreceptor Function. *Cell Rep.* 2015;10(3):370-382.
3 doi:10.1016/J.CELREP.2014.12.042
- 4 56. Kambrun C, Roca-Lapirot O, Salio C, Landry M, Moqrigh A, Le Feuvre Y. TFAFA4 Reverses
5 Mechanical Allodynia through Activation of GABAergic Transmission and Microglial
6 Process Retraction. *Cell Rep.* 2018;22(11):2886-2897. doi:10.1016/J.CELREP.2018.02.068
- 7 57. Vrontou S, Wong AM, Rau KK, Koerber HR, Anderson DJ. Genetic identification of C fibres
8 that detect massage-like stroking of hairy skin in vivo. *Nature.* 2013;493(7434):669-673.
9 doi:10.1038/nature11810
- 10 58. Liu Q, Vrontou S, Rice FL, Zylka MJ, Dong X, Anderson DJ. Molecular genetic visualization
11 of a rare subset of unmyelinated sensory neurons that may detect gentle touch. *Nat*
12 *Neurosci.* 2007;10(8):946-948. doi:10.1038/nn1937
- 13 59. Dawes JM, Weir GA, Middleton SJ, et al. Immune or Genetic-Mediated Disruption of
14 CASPR2 Causes Pain Hypersensitivity Due to Enhanced Primary Afferent Excitability.
15 *Neuron.* 2018;97(4):806-822.e10. doi:10.1016/j.neuron.2018.01.033
- 16 60. Mishra SK, Tisel SM, Orestes P, Bhangoo SK, Hoon MA. TRPV1-lineage neurons are
17 required for thermal sensation. *EMBO J.* 2011;30(3):582-593.
18 doi:10.1038/emboj.2010.325
- 19 61. Cowie AM, Moehring F, O'Hara C, Stucky CL. Optogenetic Inhibition of CGRP α Sensory
20 Neurons Reveals Their Distinct Roles in Neuropathic and Incisional Pain. *J Neurosci.*
21 2018;38(25):5807-5825. doi:10.1523/JNEUROSCI.3565-17.2018
- 22 62. Cole J, Bushnell MC, McGlone F, et al. Unmyelinated tactile afferents underpin detection
23 of low-force monofilaments. *Muscle Nerve.* 2006;34(1):105-107. doi:10.1002/mus.20534
- 24 63. J K, M H, E A, G C-B, P E. An Atlas of Vagal Sensory Neurons and Their Molecular
25 Specialization. *Cell Rep.* 2019;27(8):2508-2523.e4. doi:10.1016/J.CELREP.2019.04.096

- 1 64. Hoffmann T, Sharon O, Wittmann J, et al. NaV1.7 and pain: contribution of peripheral
2 nerves. *Pain*. 2018;159(3):496-506. doi:10.1097/j.pain.0000000000001119
- 3 65. Ackerley R, Backlund Wasling H, Liljencrantz J, Olausson H, Johnson RD, Wessberg J.
4 Human C-tactile afferents are tuned to the temperature of a skin-stroking caress. *J*
5 *Neurosci*. 2014;34(8):2879-2883. doi:10.1523/JNEUROSCI.2847-13.2014
- 6 66. Bourinet E, Martin M, Huzard D, Jeanneteau F, Mery P-F, François A. The impact of C-
7 Tactile Low threshold mechanoreceptors on affective touch and social interactions in
8 mice. *bioRxiv*. January 2021:2021.01.13.426492. doi:10.1101/2021.01.13.426492
- 9 67. Morrison I. Keep Calm and Cuddle on: Social Touch as a Stress Buffer. *Adapt Hum Behav*
10 *Physiol*. 2016;2(4):344-362. doi:10.1007/s40750-016-0052-x
- 11 68. Tigerholm J, Petersson ME, Obreja O, et al. Modeling activity-dependent changes of
12 axonal spike conduction in primary afferent C-nociceptors. *J Neurophysiol*.
13 2014;111(9):1721-1735. doi:10.1152/jn.00777.2012
- 14 69. Campero M, Bostock H, Baumann TK, Ochoa JL. Activity-dependent slowing properties of
15 an unmyelinated low threshold mechanoreceptor in human hairy skin. *Neurosci Lett*.
16 2011;493(3):92-96. doi:10.1016/j.neulet.2011.02.012
- 17 70. Roger Watkins XH, Wessberg J, Backlund Wasling H, et al. RAPID REPORT Sensory
18 Processing Optimal delineation of single C-tactile and C-nociceptive afferents in humans
19 by latency slowing Optimal delineation of single C-tactile and C-nociceptive afferents in
20 humans by latency slowing. *J Neuro-physiol*. 2017;117:1608-1614.
21 doi:10.1152/jn.00939.2016.-C-mechanoreceptors

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1 **Figure legends**

2 **Figure 1 SCN9A LOF participants perceive affective brush stimuli as less pleasant. (A)** A
 3 schematic outlining the two cohorts of participants that were recruited, the affective touch
 4 paradigm used and the self-report visual analogue scale (VAS) outcome measure. **(B)** Touch
 5 pleasantness ratings across five stroking velocities in CIP participants with SCN9A mutations and
 6 healthy control participants. CIP participants find affective brush stimuli significantly less
 7 pleasant compared to healthy controls. A 2x5 repeated measures ANOVA revealed a significant
 8 main effect of speed ($F(2.5, 226.3) = 13.55, P < 0.001, \eta_p^2 = 0.13$), a significant speed*group
 9 interaction ($F(2.5, 226.3) = 3.80, P = 0.02, \eta_p^2 = 0.04$), and a trend-level group effect ($F(1,90) =$
 10 $3.46, P = 0.07, \eta_p^2 = 0.04$). Post-hoc Mann-Whitney revealed a significant difference for 0.3 cm
 11 s^{-1} ($U = 117, P = 0.03$), 1 cm s^{-1} ($U = 95, P = 0.01$) and a trend for 3 cm s^{-1} ($U = 149, P = 0.08$). For
 12 10 cm s^{-1} and 30 cm s^{-1} results were not significant ($U = 252, P = 0.92$ for 10 cm s^{-1} , $U = 247, P =$
 13 0.86 for 30 cm s^{-1}). * $P < 0.05$. All data represented as median \pm quartiles.

14 **Figure 2 SCN9A LOF participants show atypical fEMG responses to affective touch stimuli. (A)**
 15 Study population, stimuli, and measurements (VAS = visual analog scale). **(B)** The average VAS
 16 ratings of pleasantness, intensity, and average facial EMG (corrugator) responses to slow (3 cm
 17 s^{-1}) or fast (30 cm s^{-1}) brush stroking on the forearm, from 8 healthy control participants and 3
 18 CIP participants. Black circles reflect mean values for male participants. Pleasantness: Healthy
 19 control participants rated slow brush as significantly more pleasant than fast brush (main effect
 20 of speed healthy: $F(1,7) = 23.8, P = 0.002, \eta_p^2 = 0.77$). CIP participants did not rate slow brush as
 21 more pleasant than fast brush (main effect of speed CIP: $F(1,2) = 0.04, P = 0.87, \eta_p^2 = 0.02$).
 22 Intensity: Healthy control and CIP participants rated fast brush as significantly more intense
 23 than slow brush (main effect of speed healthy: $F(1,7) = 9.21, P = 0.02, \eta_p^2 = 0.57$; main effect of
 24 speed CIP: $F(1,2) = 24.87, P = 0.04, \eta_p^2 = 0.92$). Corrugator: In healthy controls there was
 25 significantly greater corrugator activity elicited from fast brush stimuli and a reduction in
 26 activity elicited by slow brush stimuli ($F(1,7) = 8.59, P = 0.02, \eta_p^2 = 0.55$). This was not observed
 27 in CIP participants ($F(1,2) = 0.31, P = 0.63, \eta_p^2 = 0.13$). Zygomatic: There was no significant effect
 28 of brushing velocity on zygomatic activity in either group (not illustrated; healthy: $F(1,7) = 3.77,$

1 $P = 0.093$); CIP participants: ($F(1,2) = 3.25$, $P = 0.21$)). (C) Trial-by-trial touch ratings and facial
 2 EMG responses for individual CIP participants. CIP participant 2, 3 and 4 all reported no
 3 difference in pleasantness between slow and fast brushing velocities (CIP 2: $t(22) = 1.46$, $P =$
 4 0.16 ; CIP 3: $U = 31$, $P = 0.9$; CIP 4: $t(14) = 1.55$, $P = 0.14$). CIP participant 2 (female) and 3 rated
 5 fast touch as more intense than slow touch (CIP 2: $t(22) = 4.21$, $P < 0.001$; CIP 3: $t(14) = 7.67$, $P <$
 6 0.001). Similarly, CIP participant 4 reported slow touch as marginally less intense than fast (CIP
 7 4: $t(14) = 1.88$, $P = 0.08$). In CIP participant 2, 3 and 4, there was no velocity-based difference in
 8 corrugator activity (CIP 2: $t(30) = 1.06$; $P = 0.30$; CIP 3: $t(28) = 0.19$; $P = 0.85$; CIP 4: $t(30) = 1.27$,
 9 $P = 0.22$) or zygomatic activity (not illustrated) (CIP 2: $t(27) = 0.015$, $P = 0.98$; CIP 3: $t(27) = 0.76$,
 10 $P = 0.45$; CIP 4: $t(30) = 0.35$; $P = 0.73$). Asterisks reflect within-group and within-subject
 11 analyses, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, # $P < 0.1$. All data represented as mean \pm SD.

12 **Figure 3 Rodent C-LTMRs express Nav1.7, which innervate hind-paw hairy and plantar skin.**

13 (A) ISH of L4 DRG sections showing Nav1.7mRNA positive cell co-localisation with TH a marker
 14 C-LTMRs. Scale bars 25 μ m. (B) 100% of TH positive C-LTMRs express Nav1.7mRNA (226/226
 15 cells from 3 mice). (C) Example of genetically labelled C-LTMRs using the TH^{CreERT2} mouse line
 16 crossed with the Cre dependent reporter (tdTomato), scale bar 50 μ m. (D) Co-localisation of
 17 genetically labelled C-LTMRs and TH antibody labelled C-LTMR cell bodies in the DRG. TH^{CreERT2}
 18 positive C-LTMRs are a largely non-overlapping population and minimal co-localisation was
 19 seen between Parvalbumin (PV) (E), IB4 (F), CGRP (G), NF200 (H). (I) Almost all TH positive C-
 20 LTMRs co-express Nav1.8 as seen by co-localisation of genetically labelled Nav1.8 afferents
 21 (Nav1.8^{Cre}TdTom) and the TH antibody. d-i scale bar 100 μ m. (J) tdTomato labelled C-LTMRs
 22 forming longitudinal lanceolate endings around hair follicles in hind-paw hairy skin. Scale bar
 23 25 μ m. (K) tdTomato positive C-LTMR innervating the hair follicles found on the plantar surface
 24 (located between the running pads) of mice. Scale bar 25 μ m. (L) TH positive C-LTMRs do not
 25 terminate in the skin as PGP9.5+ epidermal free nerve endings. Scale bar 50 μ m.

26 **Figure 4 The genetic loss of Nav1.7 in rodent C-LTMRs results in mechanical hyposensitivity**
 27 **and abnormalities in cooling detection.** Acute sensory testing of mice lacking Nav1.7 in the TH
 28 positive C-LTMR population (Blue – TH^{CreERT2}:Nav1.7-KO) compared to controls (Grey – Nav1.7-

1 WT). (A) C-LTMR specific deletion of Nav1.7 results in significant hyposensitivity to punctate
 2 mechanical von Frey stimuli (WT $n = 31$ mice, KO $n = 28$ mice, two-tailed Student's unpaired t-
 3 Test, $t(57) = 5.062$, $P < 0.0001$, ***). (B) The number of responses to a cotton swab or (C) to a
 4 brush are not affected by the loss of Nav1.7 in rodent C-LTMRs (WT $n = 19$ mice, KO $n = 19$
 5 mice, two-tailed Student's unpaired t-Test, $t(36) = 0.690$, $P = 0.49$ and $t(36) = 1.09$, $P = 0.28$,
 6 respectively, n.s). (D) Mice specifically lacking Nav1.7 in C-LTMRs show no changes in their
 7 latency to withdraw from a noxious 53°C hotplate. (WT $n = 19$ mice, KO $n = 19$ mice, two-tailed
 8 Student's unpaired t-Test, $t(36) = 0.470$, $P = 0.64$, n.s). (E) Mice lacking Nav1.7 in C-LTMRs spend
 9 more time in cooler zones during 0-30 minutes of the thermal gradient test. The non-linear
 10 regression Gaussian fitted curves are significantly different between WT and KO mice, with KO
 11 mice showing a leftward shift toward colder temperatures (WT $n = 12$ mice, KO $n = 9$ mice, non-
 12 linear regression F-Test, $F(3, 351) = 12.95$ $P = < 0.0001$, ***). (F) The preferred temperature
 13 (the average temperature at which most time was spent) is significantly lower in KO mice
 14 compared to WT mice during 0-30 minutes of a thermal gradient test (WT $n = 12$ mice, KO $n = 9$
 15 mice, two-tailed Student's unpaired t-Test, $t(19) = 2.689$, $P = 0.0145$, *). All data represented as
 16 mean \pm SEM.

17 **Figure 5 Rodent C-LTMRs that lack Nav1.7 show smaller sodium currents and hypo-**
 18 **excitability.** (A) TH^{CreERT2} (control) or TH^{CreERT2}Nav1.7^{lox/flox} (TH^{CreERT2}Nav1.7-KO) mice received
 19 and intrathecal injection of AAV.Flex.eGFP to target C-LTMRs prior to tamoxifen administration.
 20 Subsequent tamoxifen injection initiated simultaneous eGFP and Nav1.7 ablation. (B) Virally
 21 targeted C-LTMRs were cultured, eGFP expression used to identify the population and voltage-
 22 clamp used to analyse sodium currents in both control and TH^{CreERT2}Nav1.7-KO mice. (C)
 23 Example traces of recorded total sodium currents in eGFP positive C-LTMRs from TH^{CreERT2} and
 24 TH^{CreERT2}Nav1.7-KO mice. (D) C-LTMRs lacking Nav1.7 have a significantly reduced sodium
 25 current density compared to control C-LTMRs (TH^{CreERT2} $n = 12$ cells, TH^{CreERT2}Nav1.7-KO $n = 11$
 26 cells. Mann Whitney U test, $U = 28$, $P < 0.018$, *). (E) Example sodium current traces from
 27 TH^{CreERT2} and TH^{CreERT2}Nav1.7-KO C-LTMRs, in order to determine the sodium current-voltage
 28 (I/V) relationship. (F) Quantification of the I/V relationship displayed as I/V curves. C-LTMRs
 29 from TH^{CreERT2}Nav1.7-KO mice show a significantly smaller I/V curve compared to C-LTMRs from

1 TH^{CreERT2} mice (TH^{CreERT2} $n = 12$ cells, TH^{CreERT2}Nav1.7-KO $n = 11$ cells. two-way ANOVA, $F(1, 432)$
2 = 24.05, $P < 0.0001$, ***, with Sidak-Holm post-hoc test, -25pA, $t(432) = 3.342$, $P = 0.021$, *). **(G)**
3 Single fibre recordings from the mouse skin-nerve (saphenous) preparation comparing
4 recordings from Nav1.7-WT (Grey) and TH^{CreERT2}:Nav1.7-KO (Blue) mice. C-LTMR conduction
5 velocities are normal and comparable between both WT and C-LTMRs lacking Nav1.7. (WT $n =$
6 14 units, KO $n = 12$ units, two-tailed Student's unpaired t-Test, $t(24) = 0.103$, $P > 0.91$, n.s.) **(H)**
7 The mechanical thresholds of TH^{CreERT2}:Nav1.7-KO C-LTMRs are significantly higher than Nav1.7-
8 WT control C-LTMRs (WT $n = 14$ units, KO $n = 12$ units, two-tailed Student's unpaired t-Test,
9 $t(24) = 4.070$, $P = 0.0004$, ***) **(I)** Example trace of evoked action potentials in response to a
10 supra-threshold mechanical stimulus applied to a single Nav1.7-WT and TH^{CreERT2}:Nav1.7-KO C-
11 LTMR receptive field. **(J)** The increasing force stimulus-response function showing that C-LTMRs
12 lacking Nav1.7 are significantly hypo-excitable to supra-threshold stimuli compared to control
13 C-LTMRs (WT $n = 14$ units, KO $n = 12$ units, two-way ANOVA, $F(1, 95) = 11.87$, $P = 0.0008$, ***)
14 **(K)** The increasing velocity stimulus-response function of Nav1.7-WT and TH^{CreERT2}:Nav1.7-KO C-
15 LTMRs. C-LTMRs lacking Nav1.7 are hypo-excitable with a significantly reduced firing frequency
16 to dynamic stimuli (WT $n = 14$ units, KO $n = 12$ units, two-way ANOVA, $F(1,96) = 6.212$, $P =$
17 0.014, *). **(L)** The 31-14°C cooling stimulus-response of Nav1.7-WT and TH^{CreERT2}:Nav1.7-KO C-
18 LTMRs. C-LTMRs lacking Nav1.7 are hypo-excitable to cooling stimuli. The linear regression
19 fitted slopes are significantly different between WT and KO mice (WT $n = 10$ units, KO $n = 8$
20 units, linear regression F-test, $F(1, 306) = 9.32$, $P = 0.0024$, **). **(M)** The 14-42°C warming
21 stimulus-response of Nav1.7-WT and TH^{CreERT2}:Nav1.7-KO C-LTMRs are similar. The non-linear
22 regression Gaussian fitted curves are not significantly different between WT and KO mice, both
23 groups and share a common curve (green) (WT $n = 10$ units, KO $n = 8$ units, non-linear
24 regression F-test, $F(3, 504) = 0.763$, $P = 0.515$, n.s.). **(N)** The 42-14°C cooling stimulus-response
25 of Nav1.7-WT and TH^{CreERT2}:Nav1.7-KO C-LTMRs. C-LTMRs lacking Nav1.7 are hypo-excitable to
26 cooling stimuli. The non-linear regression Gaussian fitted curves are significantly different
27 between WT and KO mice (WT $n = 10$ units, KO $n = 8$ units, non-linear regression F-test, $F(3,$
28 508) = 5.106, $P = 0.0017$, **). All data represented as mean \pm SEM.

1 **Figure 6 Selective small molecule inhibition of Nav1.7 currents results in hypo-excitable C-**
2 **LTMR sensory endings.** (A) DRG neuronal cultures were made from genetically labelled C-
3 LTMRs (TH^{CreERT2}Ai32/eYFP) and used for subsequent voltage clamp analysis. (B) Top, During the
4 voltage-clamp protocol, neurons were depolarised to -75mV from a holding potential of -
5 120mV for 8s (to inactivate a proportion of Nav1.7 channels), followed by a 2ms recovery step
6 to -120mV and a test pulse to 0 mV. Bottom, example traces of total sodium currents induced
7 during the test pulse (shaded region of protocol schematic) in the presence of vehicle or PF-
8 05089771 (10nM). (C) Quantification of the sodium current densities in wildtype C-LTMRs in the
9 presence of vehicle or PF-05089771. Blockage of Nav1.7 using PF-05089771 in C-LTMRs results
10 in a significant reduction in the sodium current density compared to vehicle treated C-LTMRs
11 (Vehicle $n = 16$ cells, PF-05089771 $n = 17$ cells, Mann Whitney U test, $U = 49$, $P = 0.0012$, **) (D)
12 Example illustration of single fibre C-LTMR recordings from wild type mice. C-LTMRs were
13 identified and subsequently recorded following a 10 minute incubation of vehicle or PF-
14 05089771 (10nM) applied directly to the isolated receptive field. (E) C-LTMR mechanical
15 thresholds pre and post vehicle or PF-05089771. Small molecule inhibition of Nav1.7 in C-LTMR
16 sensory endings results in a significant increase in the mechanical threshold compared to
17 vehicle. (Vehicle $n = 10$ units, PF-05089771 $n = 9$ units, repeated measures two-way ANOVA $F(1,$
18 $17) = 12.66$, $P = 0.0024$, **, with Bonferroni post-hoc tests, vehicle pre vs post: $t = 0.151$, $P = >$
19 0.99 , n.s, PF-05089771 pre vs post, $t = 5.82$, $P = <0.0001$, ***). (F) The increasing force stimulus-
20 response function showing that PF-05089771 treated C-LTMRs are significantly hypo-excitable
21 to supra-threshold stimuli compared to vehicle treated C-LTMRs (Vehicle $n = 10$ units, PF-
22 05089771 $n = 9$ units, two-way ANOVA, $F(1, 68) = 6.95$, $P = 0.0104$, *). All data represented as
23 mean \pm SEM.

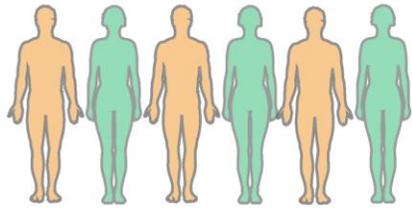
24 **Figure 7 Modelling of human SCN9A mutations in a C-LTMR computational model shows**
25 **hypo-excitability.** (A) Computational modelling of healthy control participant C-LTMRs with no
26 mutations in Nav1.7. Example traces of C-LTMR excitability and firing patterns assessed by
27 increasing the current injected into the model. Examples of C-LTMR excitability and firing
28 patterns when the model was adapted to take into account the changes in Nav1.7 conductance
29 due to the following SCN9A mutations; (B) FS1773, (C) G1725R, (D) R896W, (E) R830X. The

1 overlaid current value denotes the threshold of each model. All SCN9A mutations have
2 increased thresholds to current injections. **(F)** The healthy control model was subsequently ran
3 to increase the current injection successively by 25pA in order to assess suprathreshold
4 excitability. The model was adapted (change in Nav1.7 conductance) to take into account each
5 SCN9A mutation **(G)** FS1773, **(H)** G1725R, **(I)** R896W, **(J)** R830X and executed to assess
6 suprathreshold excitability. **(K)** The merge of all models clearly illustrates that all SCN9A
7 mutation models are hypo-excitabile and require much larger current injections in order to
8 repetitively fire to the same frequency as the healthy control C-LTMR model.

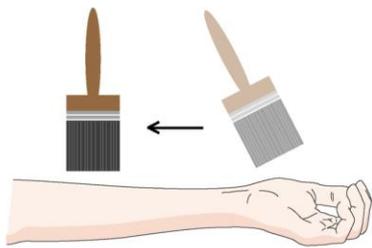
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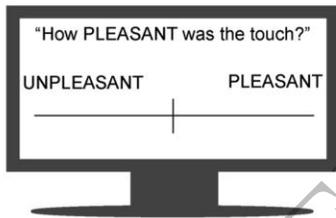
A 86 healthy participants
6 Scn9a-LOF CIP participants



Range of stimulus velocities
(0.3cm/s - 30cm/s)



Outcome measures
Self report (visual analogue scale)



B

Pleasantness rating

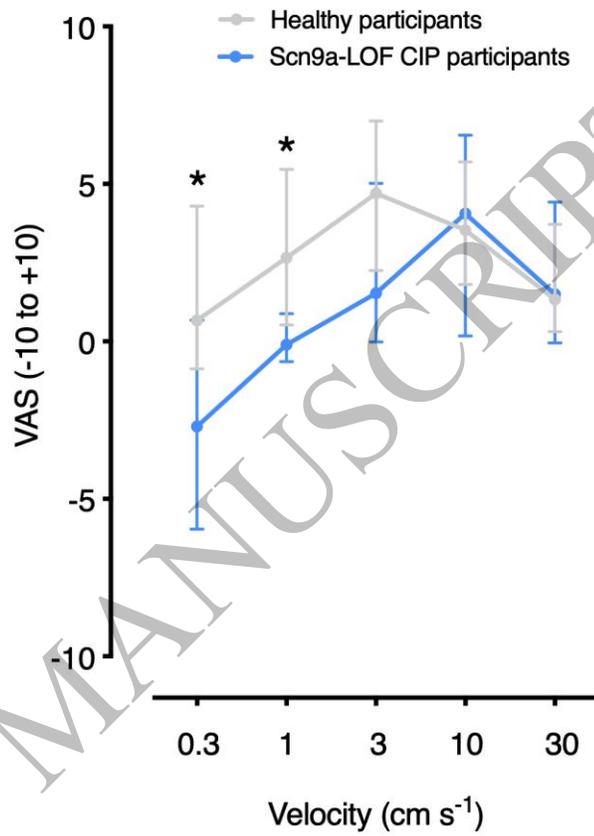


Figure 1
165x147 mm (.00 x DPI)

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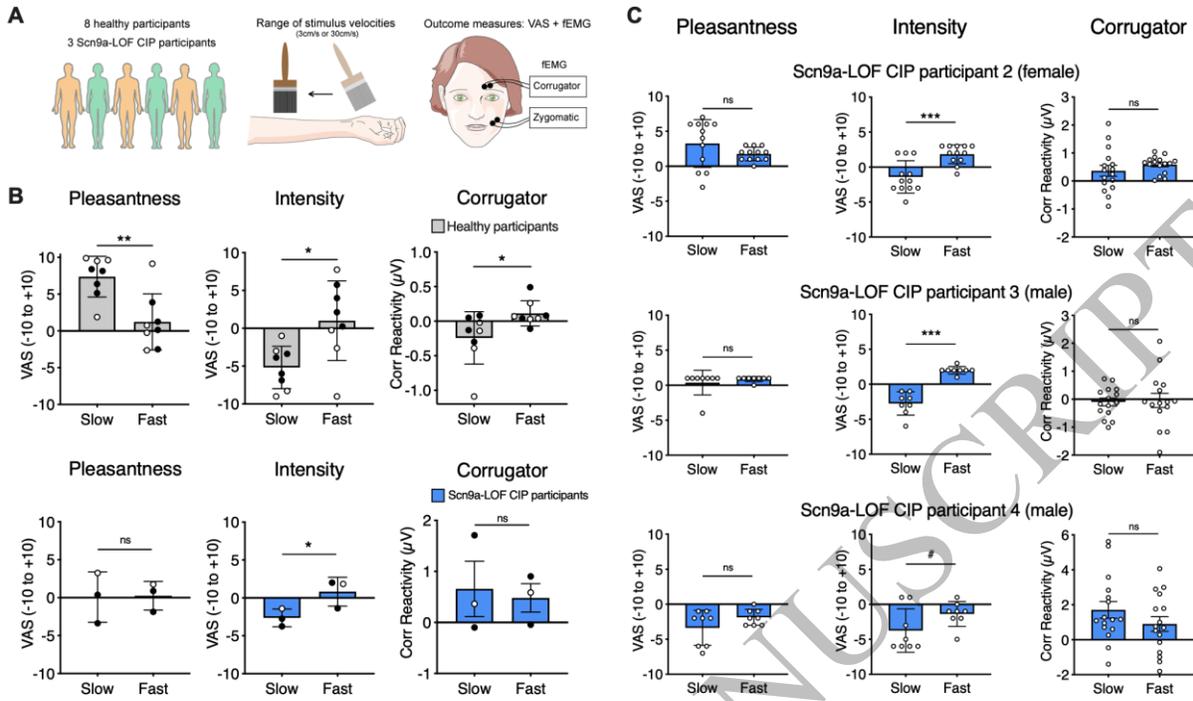


Figure 2
165x102 mm (.00 x DPI)

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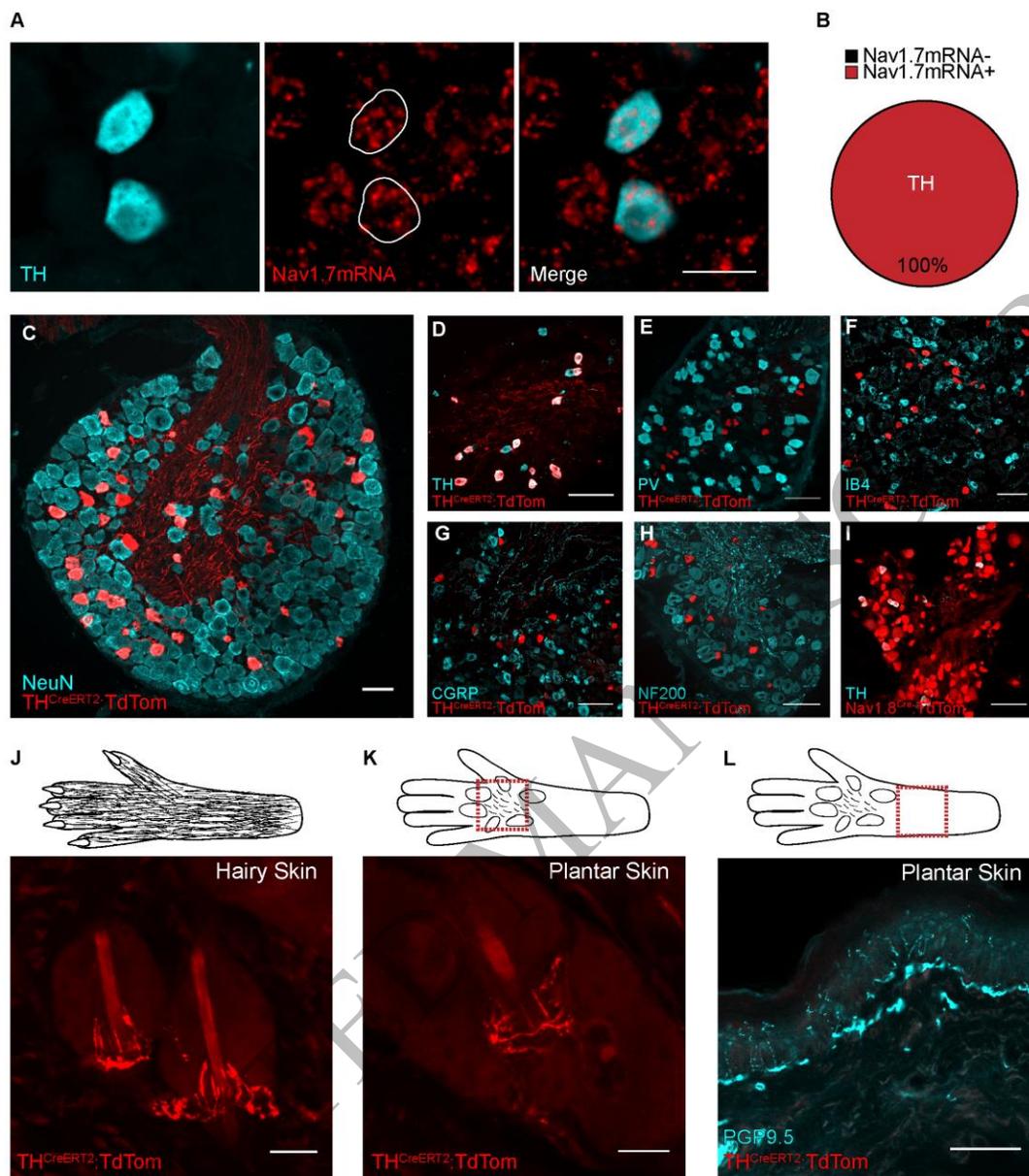


Figure 3
165x160 mm (.00 x DPI)

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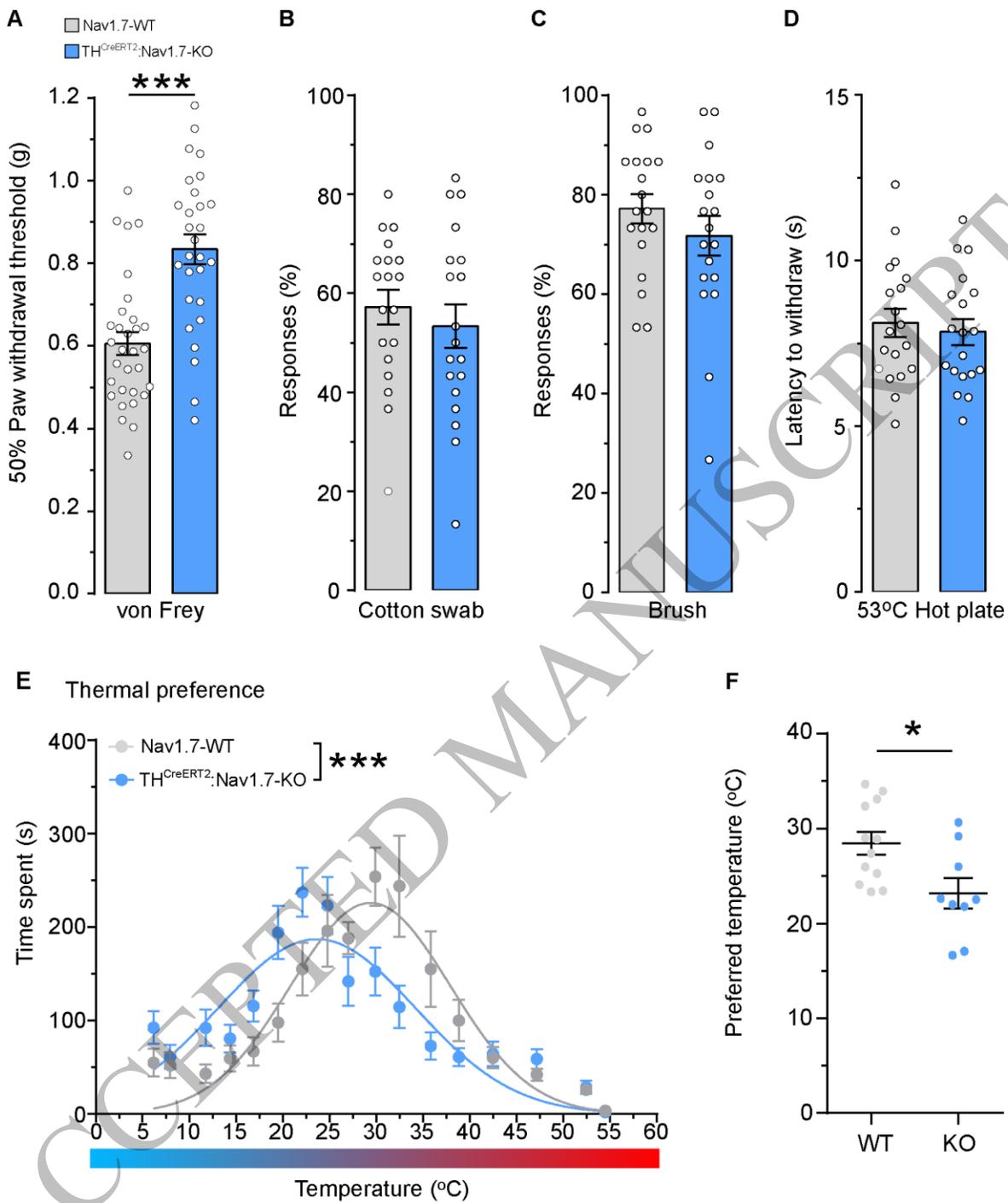


Figure 4
156x187 mm (.00 x DPI)

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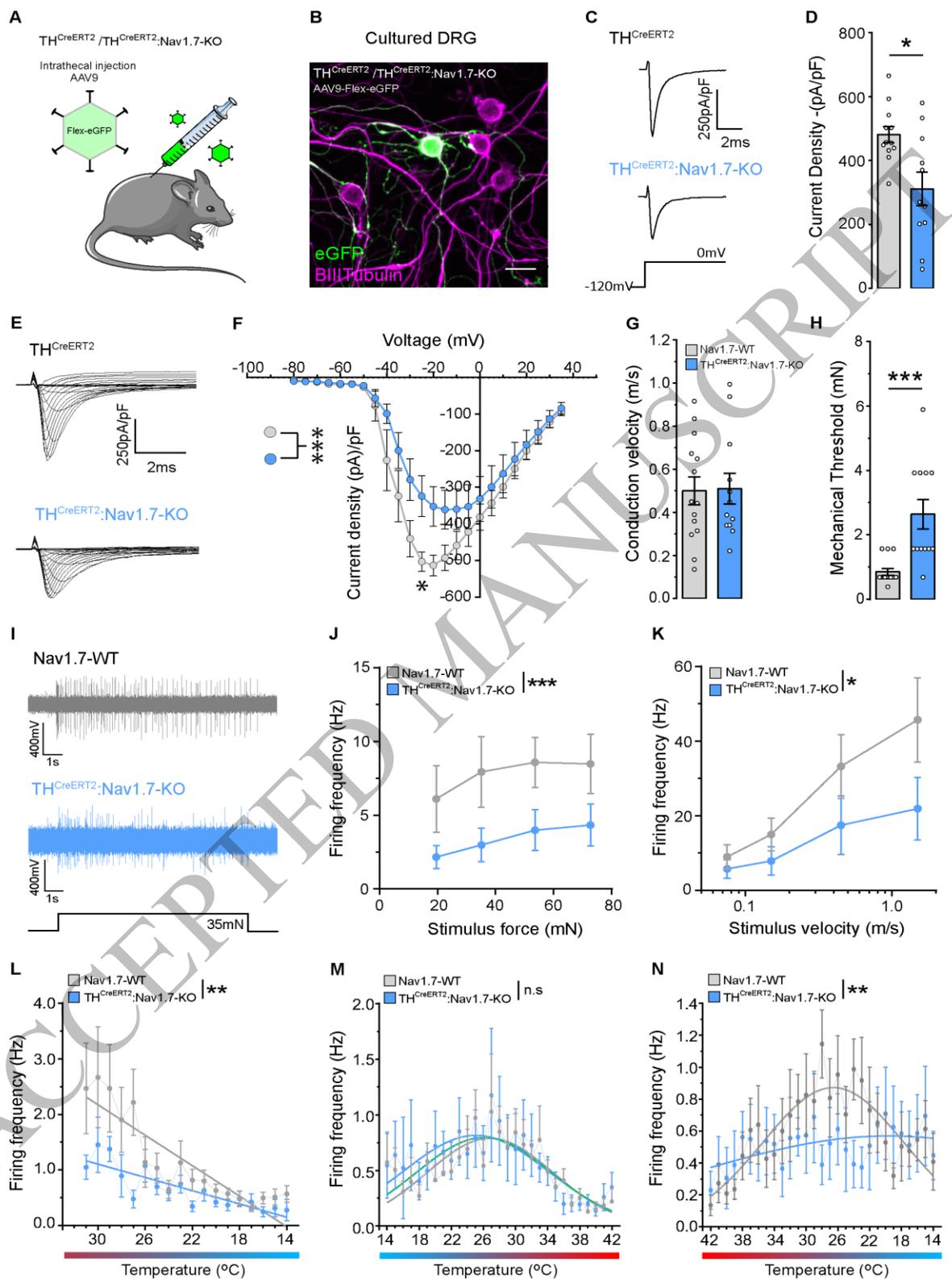


Figure 5
163x229 mm (.00 x DPI)

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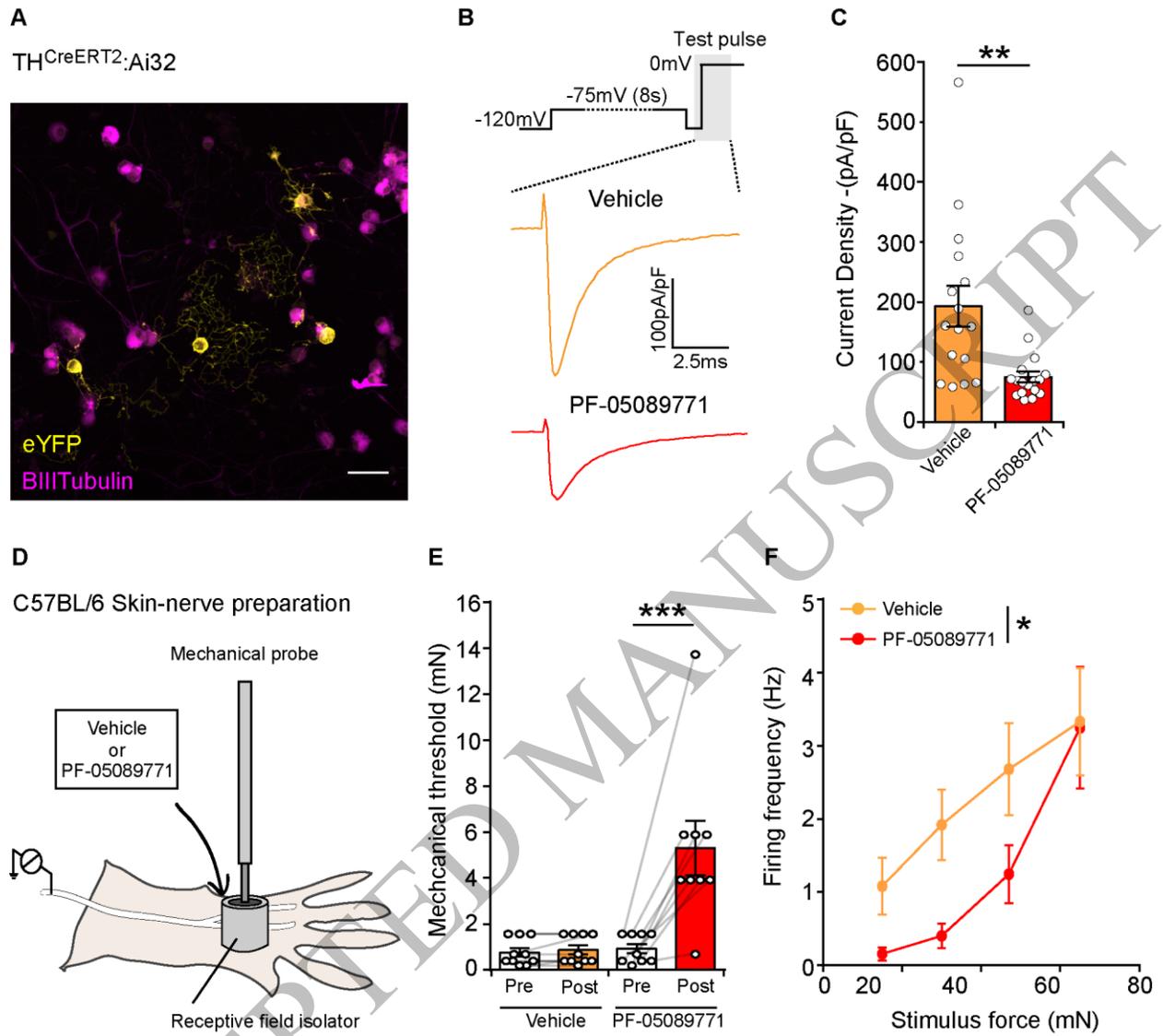


Figure 6
165x149 mm (.00 x DPI)

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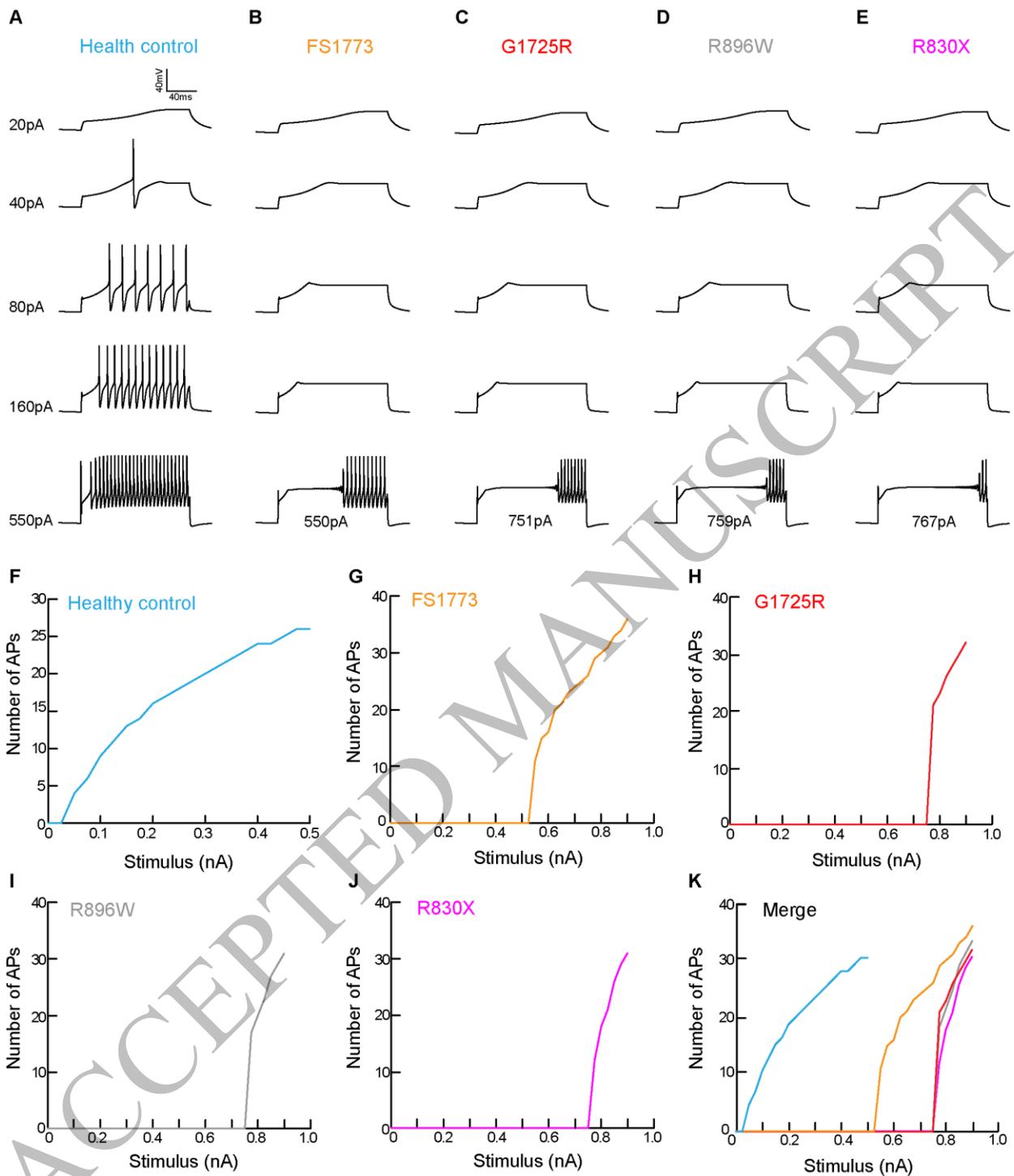


Figure 7
165x192 mm (.00 x DPI)

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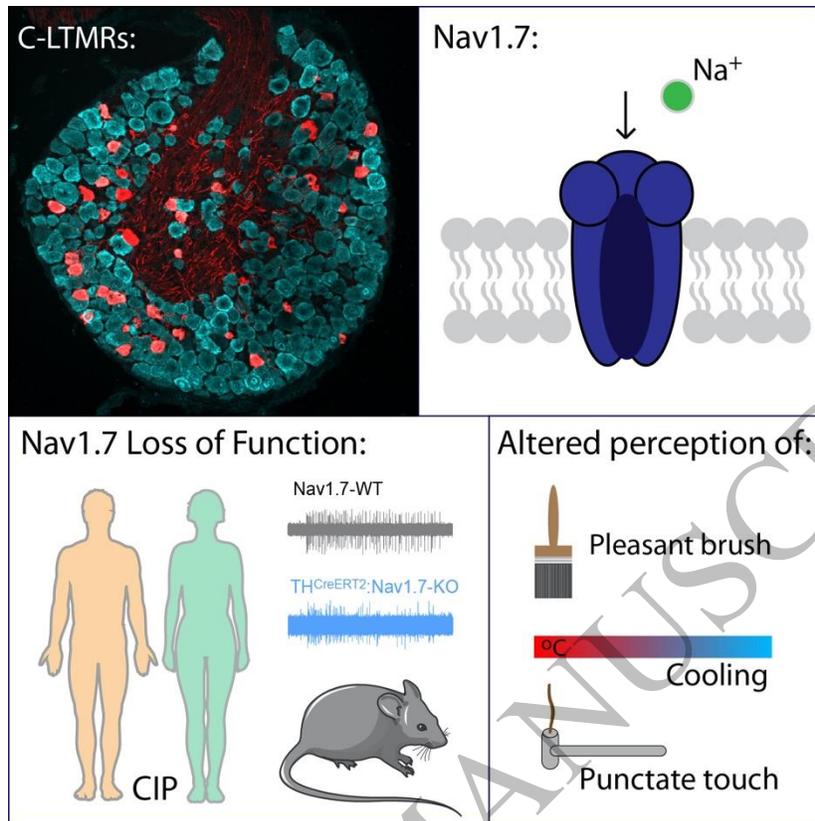


Figure 8
109x109 mm (.00 x DPI)

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1 Middleton, Perini *et al.* show that the role of Na_v1.7 extends beyond pain perception. Using a
2 multidisciplinary, cross-species approach, they show that Na_v1.7 is also essential for C-low
3 threshold mechanoreceptor function in mice and humans, regulating pleasant touch, punctate
4 discrimination and sensitivity to cooling.

5

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