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Investigating genotype-phenotype relationship of extreme neuropathic pain disorders in a national cohort: 'NIHR Bioresources Rare Disease – Neuropathic Pain Disorders'

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Abstract

Objective: ~~By integrating whole genome sequencing into healthcare~~ The aims of our study were to use whole genome sequencing in a cross-sectional cohort of patients ~~we aimed~~ to identify new variants in genes implicated in neuropathic pain, to determine prevalence of known pathogenic variants, and understand the relationship between pathogenic variants and clinical presentation.

Methods: Patients with extreme neuropathic pain phenotypes (both sensory loss and gain) were recruited from secondary care clinics in the UK, and underwent whole genome sequencing as part of NIHR-Bioresource. A multi-disciplinary team assessed pathogenicity of rare variants in genes previously known to cause neuropathic pain disorders and exploratory analysis of research candidate genes was completed. Association testing for genes carrying rare variants was completed using the gene-wise approach of the combined burden and variance-component test SKAT-O. ~~Electrophysiological analysis~~ Patch clamp analysis was performed on transfected HEK293T cells ~~was completed~~ for research candidate variants of genes encoding ion channels.

Results:

1) Medically actionable variants were found in 12% of study participants (205 recruited), including known pathogenic variants: *SCN9A(ENST00000409672.1):* c.2544T>C, p.Ile848Thr ~~p.I848T~~ that causes inherited erythromelalgia, and *SPTLC1(ENST00000262554.2):*c.340T>G, p.Cys133Tr ~~p.C133W~~ variant that causes hereditary sensory neuropathy type-1.

2) Clinically relevant variants were most common in voltage-gated sodium channels.

3) *SCN9A(ENST00000409672.1):*c.554G>A, pArg185His ~~p.R185H~~ variant was more common in non-freezing cold injury participants than controls, and causes a gain of function of $Na_V1.7$ after cooling (the environmental trigger for non-freezing cold injury).

4) Rare variant association testing showed a significant difference in distribution for genes NGF, *KIF1A*, *SCN8A*, *TRPM8*, *KIF1A*, *TRPA1* and the regulatory regions of genes *SCN11A*, *FLVCR1*, *KIF1A*, and *SCN9A* between European participants with neuropathic pain and controls.

5) The *TRPA1(ENST00000262209.4):c.515C>T,* ~~p.A172V~~ p.Ala172Val variant identified in participants with episodic somatic pain disorder demonstrated gain of channel function to agonist stimulation.

Conclusion: Whole genome sequencing identified clinically relevant variants in over 10% of participants with extreme neuropathic pain phenotypes. The majority of these variants were found in ion channels. Combining genetic analysis with functional validation can lead to better understanding as to how rare variants in ion channels lead to sensory neuron hyper-excitability, and how cold, as an environmental trigger, interacts with the gain of function $Na_V1.7$ p.Arg185His ~~R185H~~ variant. Our findings highlight the role of ion channel variants in the pathogenesis of extreme neuropathic pain disorders, likely mediated through changes in sensory neuron excitability and interaction with environmental triggers.

Introduction

Neuropathic pain occurs as a consequence of a disease or lesion in the somatosensory nervous system (Treede *et al.*, 2008). It affects 6.9-10% of the general population (van Hecke *et al.*, 2014) and has a harmful impact on physical health, psychological health and quality of life (Attal *et al.*, 2011). Understanding the role of genetic factors in neuropathic pain may reveal new pathophysiological mechanisms and is under-explored (Hébert *et al.*, 2017; Calvo *et al.*, 2019).

Extreme pain phenotypes, caused by rare high-impact genetic mutations, offer insight into fundamental neurobiological mechanisms of pain (Bennett and Woods, 2014). The phenotypes can range from congenital insensitivity to pain (Schon *et al.*, 1993) to enhanced pain perception. Different types of mutations in the same gene can cause a spectrum of phenotypes. For example, biallelic loss of function mutations in *SCN9A*, the gene encoding the voltage-gated sodium channel (Na_v) 1.7 and which is highly expressed in peripheral sensory neurons (Bennett *et al.*, 2019), causes congenital insensitivity to pain (Cox *et al.*, 2006). In contrast, monoallelic gain of function variants in the same gene are associated with pain disorders, which are inherited in a Mendelian fashion. These include inherited erythromelalgia (Yang *et al.*, 2004) and paroxysmal extreme pain disorder (Fertleman *et al.*, 2006).

Variants in genes causing Mendelian pain disorders may act as risk factors for common acquired neuropathic pain disorders. For example, *SCN9A* variants are implicated in more common neuropathic pain disorders such as idiopathic small fibre neuropathy (Faber *et al.*, 2012a) and painful diabetic neuropathy (Blesneac *et al.*, 2018). The *SCN9A* NM_002977.3:c.3448C>T, ~~p.R1150W~~p.Arg1150Trp variant modulates risk and severity of pain across different chronic pain disorders (Reimann *et al.*, 2010). An environmental trigger that interacts with genes may cause neuropathic pain as some variants are common in the general population. Identification of such gene variants is important in diagnosis, genetic counselling and treatment enabling a stratified approach, such as the use of sodium channel blockers (e.g. lacosamide) in patients with small fibre neuropathy and gain-of-function Na_v 1.7 variants (Labau *et al.*, 2020). In some cases, such as inherited erythromelalgia, a personalised management approach can be used (Yang *et al.*, 2012; Geha *et al.*, 2016). Furthermore, there are rare inherited conditions where direct treatment can arrest progression, such as Fabry's disease and hereditary transthyretin amyloidosis. In these diseases neuropathic pain is often the first symptom, the disease will progress if untreated and timely genetic diagnosis is essential to initiate appropriate treatment (Carroll *et al.*, 2022).

The National Institute of Health Research (NIHR) BioResource Rare Disease project applied whole genome sequencing to a range of rare diseases, including Neuropathic Pain Disorders (Turro *et al.*, 2020). The aims of our study were to aid the genetic diagnosis of patients with extreme neuropathic pain phenotypes, to determine the prevalence of gene variants associated with Neuropathic Pain Disorders, and to understand how the functional changes caused by gene variants relate to clinical presentation and neuropathic pain (supplementary figure 1).

Methods

Recruitment and clinical phenotyping of participants

We recruited patients with extreme neuropathic pain phenotypes, both sensory loss and gain, from secondary care clinics in the UK, located in Oxford, London, Salford, and Newcastle. Study participants with a history of lifestyle altering sensory disorder, either pain or loss of sensation, for greater than three months were invited to participate. The criteria for clinical case definitions are shown in table 1 (Turro *et al.*, 2020). We excluded patients with a known underlying genetic cause of chronic pain e.g. Fabry's disease and *SCN9A* congenital erythromelalgia (genetic pre-screening for these disorders was not mandatory), pregnancy, coincident major psychiatric disorders, poor or no English language skills, patients with documented central nervous system lesions, or patients with insufficient mental capacity to provide informed consent or to complete phenotyping. Description of the clinical phenotyping can be found in the summary NIHR Bioresource paper by Turro et al. (Turro *et al.*, 2020), and is briefly described here.

Study participants attended a single appointment that included a clinical assessment, screening for neuropathic pain, and specialised investigations to investigate for a distal symmetrical polyneuropathy. A detailed medical and drug history was taken, followed by a structured upper and lower limb neurological examination to detect clinical signs of a distal symmetrical polyneuropathy (Kleyweg *et al.*, 1991; Medical Research Council - Nerve Injuries Research Committee, 2010). DN4 questionnaire was used as a screening tool for neuropathic pain (Bouhassira *et al.*, 2005). Confirmatory tests included nerve conduction studies (England *et al.*, 2005), skin biopsy for intraepidermal nerve fibre density (Lauria *et al.*, 2010a; Lauria *et al.*, 2010b) and thermal thresholds in the area of neuropathic pain (Devigili *et al.*, 2019). Study participant's pain was assessed and graded (supplementary figure 2) according to published guidelines (Finnerup *et al.*, 2016).

Whole-blood samples were collected and sent to the NIHR BioResource laboratory in Cambridge. Detailed description of the DNA sequencing, WGS data-processing pipeline and identification of relevant gene variants can be found in Turro et al. (Turro *et al.*, 2020) and relevant aspects are summarised below.

All participants provided written informed consent in accordance with the Declaration of Helsinki. The study was approved by the East of England Cambridge South national research ethics committee (REC) reference 13/EE/0325.

Analysis plan

Genetic analysis was completed in two parts. The first analysis was to identify variants of clinical relevance in known pain genes. ~~The second was a gene-wise association analysis~~, grouping rare variants in genes for all neuropathic pain phenotypes considering both a targeted panel of pain genes and their promoters and all genes in the human genome that carried rare

variants. Two ion channel variants were selected for electrophysiological analysis to investigate their functional impact (supplementary figure 1).

Clinical reporting of pertinent findings

Gene list and transcript selection

A list of genes separated into three tiers were curated at the time of recruitment in 2015 and updated in 2021 (table 2). The division was based on the strength of evidence for the gene being linked to neuropathic pain (Turro *et al.*, 2020). Only Tier 1 genes were discussed in the multi-disciplinary team meetings and considered for clinical reporting.

Variant filtering to identify variants of clinical relevance

Variants of the 14 Tier 1 Neuropathic Pain Disorders genes were prioritised based on
(i) Minor Allele Frequency (MAF) in gnomAD < 1/1,000 if variant not previously described in association with disease, or < 25/1,000 if the variant was present as disease-causing or questionable disease-causing in Human Gene Mutation Database (HGMD).
(ii) predicted impact according to Ensembl Variant Effect Predictor werewas "High", "Moderate", or "splice region variant". Variants with more than three alternate alleles or an internal MAF > 10% were discarded to guard against errors in repetitive regions and prevent potential systematic artefacts (Turro *et al.*, 2020).

Variant interpretation in multi-disciplinary teams meeting

Prioritised variants were assessed by a multi-disciplinary team (Turro *et al.*, 2020). Pathogenicity assignment was ascribed according to guidelines of The American College of Medical Genetics (Richards *et al.*, 2015). Variants were classified as pathogenic, likely pathogenic, variant of uncertain significance (VUS), likely benign, or benign. Clinically relevant variants were those deemed medically actionable i.e. variants that could result in specific, defined medical recommendations, and were reported to the referring clinician.
Pathogenic, and likely pathogenic variants were deemed clinically relevant.
VUS were deemed clinically relevant if:
1) *in vitro* functional studies showed that the variant altered function but the relationship to clinical phenotype was not clear, particularly if a significant gene and environment interaction was suspected; or
2) *in silico* analysis of the variant suggested a significant effect on function as defined by
- variant position: in a functionally important domain; located in a highly evolutionary conserved section; significant biochemical consequences of amino acid exchange; affecting protein structure.
- minor allele frequency
-concordant pathogenicity through multiple *in silico* algorithms such as SIFT, Ppolyphen and Align GDVD.
VUS that did not meet the above criteria, likely benign and benign variants were not reported.

Group-wise Rare Variant Association Testing

Genetic association testing in genes carrying rare variants was carried out using the gene-wise approach of the combined burden and variance-component test SKAT-O (Lee *et al.*, 2012a; Lee *et al.*, 2012b). The SKAT-O test combines a standard gene burden test that maximises power under the assumption that all rare variants collapsed in a specific gene region are causal and acting in the same direction towards the phenotype and the sequence kernel association that calculates the weighted sum of squares of the variant score statistics and thus is more robust to the presence of variants with opposing effects. The combined SKAT-O test calculates a linear combination of the burden and variant-component test. Parametric bootstrap was used to resample residuals under the null model. The same resampled bootstrap phenotypes, considering covariates, were used for each gene. The weighting of the linear combination is optimised from the data itself. The Rho statistic indicates the weighting of each test and gives an indication of the causality and directionality of the variants' effects, with rho=1 reducing SKAT-O to a burden test (high percentage of causality in the same direction) and a rho=0 to a SKAT (causal and non-causal variants with opposing directions). Age, sex and the three first Principal Components of genetic variation were used as covariates. Only un-related individuals were used in the group-wise rare-variant analysis. Analysis was done per ethnicity and neuropathic pain clinical phenotype.

Data filtering and pre-process

Only participants with neuropathic pain were included for gene-wise rare variant association testing. In total 39 participants were excluded from the original cohort of 205 for the SKAT-O analysis (8 with no neuropathic pain; 2 age not available; 7 sequencing by Genomics England not available as mapped to Grch38 genome assembly and joint genotyping not conducted; 4 as only founders from families included). Exclusions were also based on ethnicity. We only considered 128 Europeans and 38 Africans with neuropathic pain as cases, 4 South-Asians and 14 people of "Other" ethnicity were not included. Unrelated individuals from the NIHR-Bioresource Rare Diseases cohort, not recruited as a part of Neuropathic Pain Disorders nor the Neuro Developmental Disorders cohort, were included as controls. Analysis for individuals with European and African ethnic origin were separated. The sample size used was 128 Europeans and 38 Africans as cases versus 5945 and 154 as ethnically matched controls respectively.

VCF files were normalised, left aligned and multi-allelic SNPs were broken down to bi-allelic using BCF-tools. Only SNPs and indels that were rare in gnomAD (MAF < 0.001), not common in the whole NIHR Bioresources Rare Diseases cohort (MAF < 0.05), and had MAF < 0.05 in cases and controls combined for each phenotype were considered. Variants should have passed the quality filters in the joint genotyping calls and be of high quality with a call rate > 99%, following HWE equilibrium (p value > 0.05). Alleles should have high average base call depth (> 10) and average genotype quality (> 20). Regulatory regions for Tier1-3 genes were downloaded using the GeneHancer resource form GeneCards. We then selected regions annotated as promoters or promoters/enhancers for the Tier 1-3 genes and were collapsed in one group associated with the respective gene. For the panel of Tier1-3 genes and their promoters we considered variants with "modifier", "moderate" and "high" impact effects. We further selected variants that were Nonsynonymous or having the EPACTS functional

annotations: Essential_Splice_Site, Normal_Splice_Site, Start_Loss, Stop_Loss, Stop_Gain, 5' UTR and 3' UTR. For the whole gene set (all genes in the human genome), we only considered variants of "moderate" or "high" Variant Effect Predictor impact on protein coding genes. Manta and Canvas software packages were used for detection of deletions>50 b pas described previously (Turro *et al.*, 2020). Due to the limited sample size and multiple sub-phenotypes we focused on protein coding genes and high and moderate impact variants for the whole gene set analysis.

Separate analysis was completed for: 36 Tier 1-3 genes (72 groups with their respective promoters); functionally validated variants in three voltage-gated sodium channels *SCN9A*, *SCN10A*, and *SCN11A* (Bennett *et al.*, 2019); and the whole gene set (approximately 20000 genes).

Functional *in vitro* studies of *SCN9A* p.Arg185His~~R185H~~ and *TRPA1* p.Ala172Val variants

Plasmids and site-directed mutagenesis

Human $Na_V1.7$ cDNA was cloned into a modified pcDNA3 expression vector containing downstream IRES and dsRED2 sequences (*SCN9A*-IRES-DsRED). Human β1 and β2 subunits were cloned into pIRES2-AcGFP (SCN1B-IRES-SCN2B-IRES-eGFP)(Cox *et al.*, 2006). Human TRPA1 cDNA was cloned into a modified pcDNA3 expression vector containing downstream IRES and dsRED2 sequences (*TRPA1*-IRES-DsRED) (Cox *et al.*, 2006). The mutations p.Arg185His~~R185H~~, p.Ala172Val~~A172V~~ and p.Asn~~N~~855Ser were introduced using QuickChange II XL site-directed mutagenesis kit (Agilent). The clones were sequenced by standard methods.

HEK293T cell culture and transfection

Human embryonic kidney HEK-293T cells were grown in a Dulbecco's modified Eagle's culture medium (DMEM/F-12, Invitrogen) containing 10% fetal bovine serum and maintained under standard conditions at 37 °C in a humidified atmosphere containing 5% CO_2. For study of the *SCN9A(ENST00000409672.1)*:c.554G>A, p.Arg185His~~p.R185H~~ variant, cells were transfected using the jetPEI™ transfection reagent (Polyplus-transfection Inc.) with either WT or mutant $Na_v1.7$ channel combined with β1 and β2 subunits (2:1 ratio). For study of *TRPA1(ENST00000262209.4)*:c.515C>T, ~~p.A172V~~p.Ala172Val and *TRPA1(NM_007332.3)*:c.2564A>G, ~~p.N855S~~p.Asn855Ser variants, cells were co-transfected with pMaxGFP (Amaxa) and either human *TRPA1* wild-type or human *TRPA1*-p.Ala172Val~~A172V~~/p.Asn~~N~~855Ser at a ratio of 1:5 to facilitate visualisation of positively transfected cells. The total amount of plasmid DNA transfected was 1.2 µg/µ~~u~~l per 35 mm dish. pMaxGFP positive cells were used as control. Cells were used 36 to 72 hours after transfection. Experiments were performed at room temperature, and repeated on three or more separate transfections.

Electrophysiology

Voltage clamp experiments were performed on transfected HEK293T cells. Whole-cell patch clamp recordings were conducted using an Axopatch 200B Amplifier, the Digidata 1550B Low

Noise Data Acquisition System and the pClamp10.6 software (Molecular Devices). Data were filtered at 5kHz and digitized at 20kHz. Capacity transients were cancelled, and series resistance compensated at 70-90% in all experiments. Cells were continuously superfused with extracellular solution or agonist-containing solutions through a common outlet. Standard extracellular solutions for the patch clamp experiments were used to study the respective *SCN9A* (Blesneac *et al.*, 2018) and *TRPA1* (Kremeyer *et al.*, 2010; Avenali *et al.*, 2014b) variants.

Electrophysiology study of SCN9A p.Arg185His~~R185H~~

The extracellular solutions contained (in mM): 140 NaCl,3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.3 with NaOH (adjusted to 320 mOsm/L with glucose). Patch pipettes were filled with an internal solution containing (in mM) 140 CsF, 10 NaCl, 1 EGTA, 10 HEPES, pH 7.3 with CsOH (adjusted to 310 mOsm/L with glucose) and had a typical resistance of 2-3MΩ. Leak currents were subtracted using a P/5 protocol, applied after the test pulse. A holding potential of -100mV and an intersweep interval of 10s was used for all the protocols. Measurements were done at 10°C, 20°C and 30°C.

Electrophysiology studies of TRPA1 p.Ala172Val~~A172V~~ and p.Asn855Ser~~N855S~~ *variants*

To study the effects of agonist-desensitization on channel activation, the extracellular solution contained (in mM): 127 NaCl, 3 KCl, 1 $MgCl_2$, 10 HEPES, 2.5 $CaCl_2$, and 10 glucose, pH 7.4 with NaOH. Osmolarity was adjusted to 310 mOsm/L with glucose. The intracellular solution contained (in mM): 135 KCl, 2 $MgCl_2$, 2 MgATP, 5 EGTA, and 10 HEPES, pH 7.4 with CsOH. Osmolarity was adjusted to 300 mOsm/L with glucose (Avenali *et al.*, 2014a).

To study the effects of intracellular calcium in channel modulation, the extracellular solution contained (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4 with NaOH. Osmolarity was adjusted to 310 mOsm/L with glucose. The intracellular solution contained (in mM): 130 KCl, 8 NaCl, 2 EGTA, 1 $MgCl_2$, 1 $CaCl_2$, 4 MgATP, 0.4 Na2GTP, pH 7.4 with CsOH. Osmolarity was adjusted 300 mOsm/L with glucose.

To investigate voltage-dependence, currents were recorded during a voltage step protocol consisting of 400ms voltage steps to test potentials ranging from –100mV to +180mV, followed by a final invariant step to -75mV (400ms) to measure tail currents. The holding potential was set at -0mV.

The voltage-dependence activation of hTRPA1 p.Ala172Val~~A172V~~ in response to mustard oil (Allyl isothiocyanate, AITC, a TRPA1 electrophilic agonist) and Menthol (non-electrophilic agonist of TRPA1) was measured. These recordings were performed in calcium-containing extracellular solution, to preserve agonist desensitization properties (Macpherson *et al.*, 2007). Perfusion with TRPA1 agonists was performed through a custom-made gravity perfusion system. Immediately after establishing the whole-cell configuration, perfusion was switched to extracellular solution for 2 min before beginning the voltage clamp recording. AITC (Sigma 377430) was dissolved in DMSO (Sigma D2650), and Menthol (Sigma M2772) in ethanol. The maximum final concentration of either DMSO or ethanol did not exceed 0.1%. The effect

of TRPA1 agonists on current-voltage curves were measured with a two voltage-step protocol, as described above. Voltage-ramps ranging from -100mV to + 100mV for 500ms, every 5 seconds, were applied to elucidate the temporal activation of hTRPA1 p.Ala172Val~~A172V~~ in response to AITC. In this case, the holding potential was set at -70mV.

Current voltage curves (I-V curves) were fitted using a combined Boltzmann and linear ohmic relationship: $I/I_{max}=G_{max}\ (V_m-E_{rev})/(1+\exp^{(V1/2-Vm)/k})$. Normalized conductance-voltage curves (activation curves) were fitted with a Boltzmann equation $G/G_{max}=1/(1+\exp^{(V1/2-Vm)/K})$ where G was calculated as follows $G=I/(V_m-E_{rev})$. Steady-state fast inactivation curves were fitted with $I_T/I_{Tmax}=1/(1+\exp^{-(V1/2-Vm)/k})$. Tail current derived voltage activation curves were fitted to the Boltzmann equation: $I_T/I_{T\ (Max)} = 1/(1 + \exp^{[(Vm - V1/2)/k]})$. In all the equations $V_{1/2}$ represents the half-activation and half inactivation membrane potentials; V_m is the membrane potential, E_{rev} the reversal potential, k the slope factor, G the conductance and I_T the current at a given V_m; G_{max} and I_{Tmax} are the maximum conductance and current respectively; R_{in} is the fraction of channels that are resistant to slow inactivation. Statistical significance was set at p =0.05 for group comparisons.

Statistical analysis

Electrophysiology data are presented as mean ± SEM. Statistical analysis for group comparisons included: Two-way ANOVA (temperature and genotype as categorical variables) for p.Arg185His, and One-way ANOVA (genotype as categorical variable) for p.Ala172Val with Sidak's multiple comparison test. Statistically significant differences were defined $p<0.05$. Frequencies of individual genetic variants were compared across groups using a two-tailed Fisher's exact test. Significance for gene-wise associations was set to the Bonferroni adjusted 0.01 threshold for the number of genes considered. Unadjusted p values are reported alongside the significance threshold.

Data availability

The genotype and phenotype data can be accessed by application to the NIHR BioResource Data Access Committee at dac@bioresource.nihr.ac.uk or by application to Genomics England Limited following the procedure outlined at https://www.genomicsengland.co.uk/about-gecip/joining-researchcommunity/.

Results

Study participants

A total of 205 study participants with extreme phenotypes were included (figure 1). Age ranged 4.8-84.3 years, and 115 (~~55.1~~56.1%) participants were men and 90 (43.9%) were women. In total 38~~x~~ participants were recruited of African descent. The majority of participants (90.2%) satisfied criteria for probable or definite chronic neuropathic pain. Participants with possible neuropathic pain (5.9%) included those with "neuropathic-type" pain (burning, stabbing, electric like shocks, dysaesthesias) in a neuroanatomically plausible distribution, but with no evidence of nerve injury on clinical examination or specialised investigations. Examples include those with episodic pain syndromes or those with burning pain of the hands and feet with a normal clinical examination and investigations. A group of participants (8, 3.9%) did not experience neuropathic pain, including participants with loss of pain sensation.

Gene variants reported – Tier 1 gene analysis.

After multi-disciplinary team discussion, 26 (12.0%) gene variants were categorised as clinically relevant: 3 pathogenic (1.4%), 2 likely pathogenic (0.9%) and 21 VUS deemed relevant for reporting (9.7%). Apart from three participants who declined consent for feedback of genetic testing, all variants were reported to the referring clinician. The results are summarised in table 3.

Three participants were diagnosed with pathogenic mutations. Two sisters with severe erythromelalgia, present since childhood, have the same pathogenic variant in *SCN9A* ~~p.I848T~~p.Ile848Thr. This variant is not present in control populations, is reported in several inherited erythromelalgia pedigrees, and causes gain of function through a hyperpolarising shift in the voltage dependence of activation (Cummins *et al.*, 2004; Yang *et al.*, 2004). A pathogenic variant in *SPTLC1* ~~p.C133W~~ p.Cys133Trp was identified in a participant diagnosed with a painful sensorimotor neuropathy, and is the commonest pathogenic *SPTLC1* mutation causing hereditary sensory neuropathy type-1 identified in UK patients (Davidson *et al.*, 2012).

Two participants diagnosed with small fibre neuropathy carried likely pathogenic variants, *SCN10A* ~~p.G1662S~~p.Gly1662Ser and *SCN11A* ~~p.C1543Y~~p.Cys1543Tyr. This conclusion was based on criteria available at the time of interpretation, low frequency in genetic databases, location in important region of the channel, high probability of affecting channel function and previous description in small fibre neuropathy (Faber *et al.*, 2012a)); however, we note that this variant is classified as likely benign in ClinVar. The remaining 21 variants reported were classified as VUS, due to our conservative approach in assigning pathogenicity.

Of the 40 participants recruited with non-freezing cold injury, six participants (7.5% allele frequency) carry the *SCN9A* ~~p.R185H~~ p.Arg185His variant, which is associated with small fibre neuropathy (Faber *et al.*, 2012a; Han *et al.*, 2012). The six participants were all of African descent (the majority from Ghana). Chronic non-freezing cold injury is an acquired painful sensory neuropathy observed almost exclusively in soldiers (Vale *et al.*, 2017). Soldiers of

African descent are disproportionately affected when compared to Caucasian soldiers. The allele frequency of p.Arg185His ~~p.R185H~~ variant is less than 1% in gnomAD (1.4% in the African/African-American population); 165 individuals carried the allele in general gnomAD population, 125 of which were Africans (166 allele counts, 1 was homozygote). Within NIHR Bioresource controls of African ancestry, p.Arg185His ~~p.R185H~~ allele frequency was 0.0065 (2 out 104 participants,1.3%). In our cohort (figure 2a), the allele was significantly more common (6 out of the 38 participants) compared to both the general gnomAD population (Fisher's Exact test, P value = 1.4*10e-7, OR = 28.17, 95%CI [9.98, 65.45]), the gnomAD African population (Fisher's Exact test, P value = 0.001, OR = 5.66, 95%CI [2, 13.03]), and NIHR Bioresource African controls (Fisher's Exact test, P value = 7.6*10e-4, OR = 13.68, 95% CI [2.41, 143.26]).

The variant, p.Arg185His ~~p.R185H~~, results in an amino acid substitution in the linker between D1/S2 and D1/S3, which lies within a voltage sensing domain of $Na_v1.7$ (figure 2b). This residue is highly conserved across all voltage-gated sodium channels. ~~The amino acid substitution is predicted to be "probably damaging" and "deleterious" according to the *in silico* tools Polyphen and SIFT.~~ Functional studies have shown that p.Arg185His ~~p.R185H~~ variant does not alter channel gating properties at room temperature; however, it was associated with enhanced resurgent currents, and increases excitability when expressed in in dorsal root ganglion neurons (Han *et al.*, 2012).

Intronic variants and the detection of deletions were included in the gene level analysis, and none were deemed medically actionable or of interest.

The impact of *SCN9A* ~~p.R185H~~ p.Arg185His variant on $Na_V1.7$ channel function is temperature dependent

Non-freezing cold injury is caused by cold environmental exposure and neuropathic pain is worsened by further cold exposure. We hypothesised that there may be an interaction between $Na_V1.7$ and an environmental trigger, such that cooling magnifies the effect of p.Arg185His~~p.R185H~~ variant on channel function. Wild type (WT) and p.Arg185His ~~p.R185H~~ $Na_V1.7$ channels were expressed in combination with β1 and β2 subunits in HEK293T cells and $Na_V1.7$ mediated currents recorded by whole cell patch clamp (Figure 2c-h). Changing the temperature from 20°C to 10°C did not significantly affect the half-activation potential of p.Arg185His ~~p.R185H~~ (Figure 2c), but significantly shifted the half-inactivation potential for steady-state fast inactivation of p.Arg185His ~~p.R185H~~ mutant compared to WT (figure 2d). Channel kinetics were slower at 10°C compared to 20°C with similar effects for WT and p.Arg185His ~~p.R185H~~ (Figure 2e). We also wanted to assess channel behaviour at higher temperatures (30°C). Increasing the temperature from 20°C to 30°C did not affect I-V curve or the steady-state inactivation of the WT or the p.Arg185His ~~p.R185H~~ (Figure 2 f, g). Faster inactivation kinetics were observed at 30°C and the change was similar for WT and ~~p.R185H~~ p.Arg185His (Figure 2h).

In conclusion, the ~~p.R185H~~ p.Arg185His mutant exhibited a depolarising shift of half-inactivation potentials at 10°C but not at 20°C or 30°C, which is a change in channel gating consistent with gain of function only at lower temperatures.

Gene-wise Rare Variant Association Testing Results

When comparing European participants against controls, gene-wise, rare variant association testing for Tiers 1-3 genes identified six genes and regulatory regions of 4 genes with significant difference in rare variant distribution (table 4). In total, 177018 genomic loci were called in Tier 1-3 genes, 132605 SNPs (93295 were singletons), 4059 insertions and 8498 deletions (8859 out of the 12557 were singletons). Variants per loci rate was 0.000046 in priority genes regions. Heterozygosity to Homozygosity ratio was 0.96, 1.35 for SNPs and 0.29 for INDELS. For European participants, gene-wise rare variant association varied according to clinical phenotype (supplementary table 1), and 20 genes and 24 regulatory regions reached Bonferroni adjusted significance for at least one phenotype. For African participants several genes showed similar associations with post-traumatic neuropathy, small fibre neuropathy and non-freezing cold injury (supplementary table 1).

For the groups of functionally validated voltage-gated sodium channel variants, a significant association of *SCN10A* with small fibre neuropathy in Europeans was found. This was driven by two gain of function variants, *SCN10A(ENST00000449082.2):c.4984G>A, p.Gly1662S~~p.G1662S~~* ((Allele frequency 0.0039 in Neuropathic Pain and 0.0005 in controls) and *ENST00000449082.2:c.1661T>C, ~~p.L554P~~ p.Leu554Pro* (Allele frequency 0 in Neuropathic Pain and 0.00008 in controls) (Table 4). These variants are reported in patients with small fibre neuropathy, cause gain of function of the $Na_V1.8$ channel and enhance dorsal root ganglion neuron excitability (Faber *et al.*, 2012b; Han *et al.*, 2014). However, both variants were characterised as likely benign in ClinVar and their allele frequencies in gnomAD suggest that they cannot be the only cause of neuropathic pain. No significant associations were found for *SCN9A*, nor *SCN11A*.

For the whole gene set analysis, 146 genes reached Bonferroni corrected significance in Europeans with neuropathic pain when compared to controls (supplementary table 2). Four of the genes, *KIF1A*, *KCNQ5*, *KCNK4* and *NOS2* are linked to human neuropathic pain.

The *TRPA1* ~~p.A172V~~ p.Ala172Val variant is associated with episodic pain and gain of channel function

Variants in Tier 2 and 3 genes were filtered, using the same approach as for Tier 1 genes to identify those that may be pathogenic. A rare TRPA1 variant, ~~p.A172V~~ p.Ala172Val (gnomAD allele frequency 0.0003), was identified in a participant who suffers from episodic widespread chronic pain with neuropathic characteristics particularly affecting the trunk. Clinical phenotype was similar to a neuropathic pain syndrome associated with a TRPA1 channelopathy (~~p.N855S~~ p.Asn855Ser variant) (Kremeyer *et al.*, 2010); however, the participant's pain was not precipitated by physiological stressors. MRI of the brain and spine, nerve conduction studies and skin biopsy of the lower leg were within age and gender appropriate reference ranges. The participant's child, carrying the same variant, was similarly affected by chronic

abdominal and pelvic pain with normal investigations. *In silico* tools, Polyphen and SIFT scores, predicted ~~p.A172V~~ p.Ala172Val to be deleterious. The variant is moderately conserved across species (Figure 3a), is situated within the ankyrin repeat domain of the protein (Figure 3b) and is a non-polar to non-polar amino acid exchange (Grantham score 64). Based on the rarity of the variant, clinical phenotype consistent with the TRPA1 Familial Episodic Pain Syndrome, positive family history and *in silico* analysis showing the variant to be in the poorly understood N-terminal ankyrin repeats, the *TRPA1* p.Ala172Val ~~p.A172V~~ variant was prioritised for functional studies. These were compared to the *TRPA1* variant ~~p.N855S~~ p.Asn855Ser which is the only variant previously linked to this disorder.

The biophysical properties of WT, p.Ala172Val ~~p.A172V~~ and ~~p.N855S~~ p.Asn855Ser hTRPA1 were compared (supplementary figure 3). In HEK293T transfected cells, TRPA1 current-voltage relationship and half-maximal activation for WT, ~~p.A172V~~ p.Ala172Val and ~~p.N855S~~ p.Asn855Ser were not statistically different. Under control conditions WT, ~~p.A172V~~ p.Ala172Val and ~~p.N855S~~ p.Asn855Ser current traces showed sustained outward rectification at positive potentials, consistent with an underlying voltage dependence of channel gating. As current density did not differ between WT and the variants, under control conditions, it is unlikely that channel trafficking is affected in ~~p.A172V~~ p.Ala172Val and ~~p.N855S~~ p.Asn855Ser channels.

Voltage-dependence activation of *hTRPA1* ~~p.A172V~~ p.Ala172Val and ~~p.N855S~~ p.Asn855Ser was measured in response to mustard oil (Allyl isothiocyanate, AITC, a TRPA1 electrophilic agonist) and Menthol (non-electrophilic agonist of TRPA1). In response to 25 µM AITC, in the presence of extracellular calcium, ~~p.A172V~~ p.Ala172Val showed a pronounced linearisation of the current-voltage relationship compared to WT in response to a two voltage-step protocol. A steeper activation curve was observed at positive potentials for both p.Ala172Val ~~p.A712V~~ and ~~p.N855S~~ p.Asn855Ser compared to WT (Figure 3c). Currents were significantly increased at positive potentials for ~~p.A172V~~ p.Ala172Val (Figure 3d) and at negative potentials for ~~p.N855S~~ p.Asn855Ser (Figure 3e). Analysis of tail currents demonstrated a significant leftward shift of voltage dependence of channel activation in the presence of AITC for both variants (Figure 3f); however, the slope of the voltage-activation curve did not significantly change. Current-voltage relationships, tested with a voltage-ramp protocol, showed an increase in current densities at positive potentials after application of 25µM AITC (figure 3g). In summary, these findings show an enhanced response of ~~p.A172V~~ p.Ala172Val to 25 µM AITC, suggesting a gain-of-function behaviour of this variant, under agonist stimulation.

Application of 100µM Menthol did not significantly change current density nor voltage sensitivity when applied to ~~p.A172V~~ p.Ala172Val or ~~p.N855S~~ p.Asn855Ser in the presence of extracellular calcium (supplementary figure 4). However, in the presence of both extracellular and intracellular calcium, significant changes were observed (Figure 4a-d). Both ~~p.A172V~~ p.Ala172Val and ~~p.N855S~~ p.Asn855Ser channels showed an increase in outward currents and voltage sensitivity in response to 100µM Menthol.

In summary, the ~~p.A172V~~ p.Ala172Val variant confers gain of function properties on TRPA1 channel in response to the agonists AITC and Menthol. For the latter, the effect was dependent on intracellular calcium.

Discussion

In this study we applied whole genome sequencing to a cohort of patients with Neuropathic Pain Disorders. We carried out two analyses. First, we identified medically actionable variants and second, we carried out group-wise associations tests at the gene level for rare variants. The diverse phenotypes ranged from congenital insensitivity to pain to painful neuropathy. Clinically relevant findings in genes associated with pain were reported in 12% of participants. The majority of clinically relevant variants were in voltage-gated sodium channels. We made new genotype-phenotype associations, such as the $Na_V1.7$ ~~p.R185H~~p.Arg185His variant which was more frequent in Africans with non-freezing cold injury. We provide new mechanistic insights showing that ~~p.R185H~~p.Arg185His interacts with cold, causing gain of function in the $Na_V1.7$ gating properties. The gain of function of ~~p.A172V~~ p.Ala172Val in TRPA1 in response to agonists extends our knowledge of painful TRPA1 channelopathies.

The study of genetic neuropathic pain disorders poses challenges in describing clinical phenotype and assigning pathogenicity to associated variants. We used the gold standard grading system, of the Neuropathic Pain Special Interest Group of IASP (Finnerup *et al.*, 2016). Such an approach works well in disorders where there is structural injury to sensory neurons. For example, in painful distal symmetrical polyneuropathy, pain and sensory signs are found in a neuroanatomically plausible distribution (meeting probable criteria), and specialised investigations confirm a lesion of the somatosensory nervous system (meeting definite criteria). However, the majority of Mendelian pain disorders are sensory neuron ion channelopathies, in which pain is episodic, with no structural injury to sensory neurons. For example, in Inherited Erythromelalgia ($Na_V1.7$) or Familial Episodic Pain Syndrome (TRPA1, $Na_V1.9$), sensory examination (between pain episodes), neurophysiology and cutaneous innervation are normal. Careful attention to clinical history is therefore essential.

We identified medically actionable variants in 12% of the participants. In our cohort, five participants carry ~~clear~~/pathogenic or likely pathogenic variants. Four pathogenic variants were in voltage-gated sodium channels, which can impact patient care. For example, a pair of sisters with Inherited Erythromelalgia carry the pathogenic *SCN9A* p.Ile848Thr~~p.I848T~~ with autosomal dominant pattern of inheritance. Only a minority of patients with Inherited Erythromelalgia possess *SCN9A* mutations (Zhang *et al.*, 2014). Identification of a genetic cause for erythromelalgia means family genetic counselling and preferential treatment with non-selective sodium channel blockers (Cregg *et al.*, 2014), which is not the standard treatment for other causes of neuropathic pain (Finnerup *et al.*, 2015). The family may also access future treatments such as selective sodium channel blockers (Alsaloum *et al.*, 2020).

A further 20 participants carry VUS in voltage-gated sodium channels that were deemed clinically relevant. Ascribing pathogenicity to ion channel variants is difficult. The majority are relatively common, exhibit subtle channel gain of function effects and likely interact with environmental factors. For example, in our cohort the *SCN9A(ENST00000409672.1)*: c.2215A>G, p.Ile739Val~~p.I739V~~ variant was found in four participants with painful

neuropathy and described previously as pathogenic (Han *et al.*, 2012). However, p.Ile739Valp.I739V is common in the general population (0.2% allele frequency), while the prevalence of small fibre neuropathy is rare (approximately 50 per 100 000 (Peters *et al.*, 2013)). It is unlikely that this variant is fully penetrant (thus pathogenic); but may act as a risk factor. Nevertheless, it is important to identify such variants for treatment selection, because Lacosamide (a sodium channel blocker) has shown efficacy in a clinical trial of patients with small fibre neuropathy and rare *SCN9A* (de Greef *et al.*, 2019) variants. Voltage clamp analyses can add valuable experimental evidence to the pathogenicity assessment that cannot be replaced by in-silico prediction tools The gold standard remains electrophysiological analysis, as *in silico* analysis is not comprehensive and variant's effect prediction software are likely to underestimate the impact of the gain-of-function effects. ; hHowever, recent advances in ion channel modelling has improved outcomes (Heyne *et al.*, 2020).

Whole genome sequencing is now integrated into the UK national health system (see https://www.genomicsengland.co.uk/) and is now considered standard of care across the UK (The 100000 Genomes Project Pilot Investigators, 2021). As of 2022 all testing at NHS genomic centres for painful neuropathies and channelopathies is via whole genome sequencing. The panel application 'Inherited neuropathies or pain disorder v1.36' was informed by the NIHR Bioresource, (100,000 genomes) and, our functional studies and includes all the tier 1 pain genes in this paper as well asand *TRPA1*. Such an approach will identify many more new variants and the findings of our study will be of direct relevance to practitioners assessing patients with neuropathic pain. Understanding how these novel variants relate to clinical phenotype presents significant challenges for clinicians. The combined expertise of our multidisciplinary teams meetings were vital in the interpretation of variant pathogenicity. Therefore, the findings of our study will be of direct relevance to practitioners assessing patients with neuropathic pain. We note that the proportion of cases in which we have found a pathogenic variant that explains the clinical pain phenotype was low (2.4%), although in a higher proportion (a further 10%) the finding was clinically actionable. In many cases this was VUS in which the relationship to the clinical phenotype could not be causally established; but could still have implications for treatment and so was reported. The low yield in solving cases at a diagnostic level partly represents the fact that neuropathic pain is monogenic in only a minority of cases. In many cases it is likely to arise from gene-environment interaction and/or multiple genes. To try and improve diagnostic yield in the future we could enrich analysis pipelines (for instance to include analysis of repeat expansions in the WGS data), expand co-segregation analysis and also functional analysis. It is not possible to test all novel variants with current patch clamp technology. –Automated prediction of pathogenicity and higher throughput functional assays should be a priority, and important next steps in the integration of whole genome sequencing into healthcare.

We were able to extend genotype-phenotype associations of *SCN9A*. The *SCN9A* p.R185Hp.Arg185His variant was more common in study participants with non-freezing cold injury. p.R185Hp.Arg185His is associated with neuropathic pain disorders, such as small fibre neuropathy (Faber *et al.*, 2012a) and painful diabetic neuropathy (Blesneac *et al.*, 2018). Non-freezing cold injury is a chronic neuropathic pain disorder caused by an acquired sensory neuropathy (Vale *et al.*, 2017) after prolonged exposure to cold and wet environments. Further

cold exposure intensifies the neuropathic pain in a similar manner to cold allodynia after platinum based chemotherapy (Bennedsgaard *et al.*, 2020). The mechanism for this cold allodynia is unknown. We show that ~~p.R185H~~p.Arg185His displays gain of function characteristics at lower temperatures. This occurs through a shift of fast inactivation at 10°C, but not at 30°C or room temperature (20°C). Pathogenic *SCN9A* variants that cause inherited erythromelalgia demonstrate temperature sensitivity at biophysical and clinical level such that warming intensifies pain and cooling is analgesic(Han *et al.*, 2007). A link therefore exists between biophysical properties of $Na_V1.7$ channel, clinical phenotype and temperature sensitivity. An increase of ~~p.R185H~~p.Arg185His variant excitability on cooling may contribute to cold allodynia in a sub-group of non-freezing cold injury patients. Another consideration is that increased activity of ~~p.R185H~~p.Arg185His at cold temperatures could injure nerves through calcium dependant excitotoxicity (Hoeijmakers *et al.*, 2012; Estacion *et al.*, 2015). In summary, p.Arg185His may contribute to clinical phenotype or increase risk for cold induced neuropathic pain; but, we cannot conclude that is causative in in isolation.

Bevimed is an inference procedure for identifying loci associated with rare hereditary disorders using Bayesian model comparison, and was used in an analysis of all cohorts of NIHR BioResource Rare Diseases (Turro *et al.*, 2020). Under different modes of inheritance there was strong evidence for 95 genes and 29 binary disease tags (cases versus controls). No diseases from our cohort were amongst the associations with strong evidence. We used SKAT-O in our cohort. Group-wise association tests can increase the power to detect associations between rare alleles and phenotypes by aggregating variant counts on the gene level that can have an impact on gene function. Neuropathic pain can arise as a complex effect of rare variants with causal effects in the same direction, which the burden test is best powered to detect, or effects with different levels of causality and direction, which the sequence kernel association test is best powered to detect. We used an approach that maximises test power by considering the combination of both the burden and sequence kernel association tests. In testing for associations for the whole gene set in Europeans with neuropathic pain, 146 genes reached statistical significance. These are gene-wise results showing a significant association of a configuration of rare alleles grouped at the gene level with certain neuropathic pain phenotypes. In most of the associations, the variant component test was better powered. This is indicated by the low Rho statistics empirically calculated from the data, and is suggestive of the presence of variants with different values of causality and opposing direction of effects towards the phenotype, i.e. both protective and deleterious variants present and driving the association. We focused on bi-allelic variants and indels of high quality, with moderate to high impact on protein coding genes, as we had a moderate sample size and did not want to diminish statistical power after Bonferroni correction. The genes *KCNK4*, *KCNQ5* and *NOS2*, were significantly associated with neuropathic pain after gene-wise rare variant association testing, These have not been previously linked to human pain at a genetic level; but, preclinical models have implicated these genes in pain pathogenesis (Noël *et al.*, 2009; Liu *et al.*, 2021; Shnayder *et al.*, 2021). *KIF1A*, a Tier 2-3 priority gene also reached genome wide significance. The association with neuropathic pain would require replication in independent cohorts and functional studies.

We undertook an analysis focusing on genes known or likely to be implicated in neuropathic pain, and we therefore used more relaxed criteria in filtering rare variants, including modifier, moderate and high impact variants. We also note that as our *in silico* variant effect predictions are limited by their tendency to under-estimate gain of function effects. Our analysis showed an association of five Tier 2 genes, including *TRPA1*, with neuropathic pain. TRPA1 is a calcium permeable non-selective cation channel that acts as a sensor of noxious external stimuli, such as mustard oil (AITC) and Menthol. The ~~p.N855S~~p.Asn855Ser TRPA1 variant is associated with familial episodic pain syndrome characterised by truncal pain triggered by physiological stress or exercise (Kremeyer *et al.*, 2010). We identified a study participant with a similar clinical phenotype of truncal pain who carried the ~~p.A172V~~ p.Ala172Val TRPA1 variant. ~~p.A172V~~ p.Ala172Val is a missense mutation in the fourth domain of the Ankyrin region. Our *in vitro* electrophysiological studies show that TRPA1 ~~p.A172V~~ p.Ala172Val variant causes gain of function in response to agonist stimulation, and provides evidence for Ca^{2+}-mediated channel activation through the Ankyrin repeat domains of TRPA1 channel.

TRPA1 consists of a large intracellular NH2 and COOH termini, with the NH2 terminus containing an elongated ankyrin repeat domain which is highly conserved. Human TRPA1 consists of 16 ankyrin repeat domains that connects to the transmembrane domains *via* a linker region. The ~~p.A172V~~ p.Ala172Val variant did not change the biophysical properties of TRPA1 channel in the naïve state, but did enhance responses to AITC and Menthol. Current density was larger for AITC when compared to Menthol. Due to difference in their electrophilic nature, agonists vary in their ability to covalently (AITC) or noncovalently (Menthol) modify the channel upon binding. This difference in channel binding might underlie different agonist responses. The ~~p.N855S~~p.Asn855Ser and ~~p.A172V~~ p.Ala172Val variants increase activation in response to agonists, although with distinct impacts on the biophysical properties of the channel. Intrinsic differences due to the positions of the variants within the channel (~~p.N855S~~p.Asn855Ser is in the transmembrane domain S4), might underlie the differences observed. Our data show that the ankyrin repeat domain is important in TRPA1 channel activation and gating functions. The ~~p.A172V~~ p.Ala172Val variant is reported at a heterozygous frequency of 0.0003 in gnomAD and individuals over 70 years of age are reportedly healthy so it is unlikely to be fully penetrant. It is more likely to act as a risk factor and contribute to the expression of neuropathic pain by altering the functional properties of nociceptive afferents; however, the drivers for channel activation (environmental versus endogenous ligands) are not known.

In our study we identified clinically relevant variants in 12% of the participants, with impact on clinical care and treatment. The majority of these variants ~~were~~ are located in ion channels which are enriched in nociceptors and environmental triggers (such as cold in the case of non-freezing cold injury) may interact and enhance the gain of function impact of such variants.

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Authorship contributions:

1) conception and design of the study – ACT, GB, IB, MC, KM, DLHB
2) acquisition and analysis of data - all authors
3) drafting a significant portion of the manuscript or figures – ACT, GB, IB, MC, KM, DLHB

Conflict of Interest/Disclosures:

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ASCR declares the following interests occurring in last 24 months: undertakes consultancy and advisory board work for Imperial College Consultants that included remunerated work for: Abide, Confo, Vertex, Pharmanovo, Lateral, Novartis, Mundipharma, Orion, Shanghai SIMR BiotechAsahi Kasei, Toray & Theranexis; owner of share options in Spinifex Pharmaceuticals from which personal benefit accrued upon the acquisition of Spinifex by Novartis in July 2015 (final payment was made in 2019); named as an inventor on patents: Rice A.S.C., Vandevoorde S. and Lambert D.M Methods using N-(2-propenyl)hexadecanamide and related amides to relieve pain. WO 2005/079771 andOkuse K. et al Methods of treating pain by inhibition of vgf activity EP13702262.0/ WO2013 110945; Member Joint Committee on Vaccine and Immunisation- varicella sub-committee; Analgesic Clinical Trial Translation: Innovations, Opportunities, and Networks (ACTTION) steering committee member; Medicines and Healthcare products Regulatory Agency (MHRA), Commission on Human Medicines - Neurology, Pain & Psychiatry Expert Advisory Group

Figure and Table legends

Figure 1

Flow diagram outlining neuropathic pain grading, study participant recruitment and summary of study participant clinical phenotype

Ten participants were excluded due to either samples not received, quality control failures or gender discrepancies.

□ - Included cases of longstanding progressive neuropathies where the presenting or predominant feature was altered sensory function and an underlying cause could not be identified

¶ - Include cases of post herpetic neuralgia, episodic pain syndromes, neuropathic pain with plausible neuroanatomical distribution but no abnormalities on examination and specialised tests, leprosy, hereditary neuralgic amyotrophy, type 1 complex regional pain syndrome, Noonan syndrome, injury to left arm (these cases were included due to severe pain which was in excess of the inciting injury).

* - Genes selected for electrophysiological characterisation

NOS- Not otherwise specified

Figure 2

a) ~~p.R185H~~p.Arg185His is more common in non-freezing cold injury than control populations.

b) Schematic of $Na_V1.7$ channel topology. R185H is represented with a red dot.

c) Normalized peak current-voltage relationship curves for the WT at 20°C (black squares, $V_{1/2}$ = -30.7 ± 1.3, k = 4.9 ± 0.4, n=15), WT at 10°C (grey dots, $V_{1/2}$ = -26.5 ± 2, k = 6.6 ± 0.6, n=14), R185H at 20°C (red triangles, $V_{1/2}$ = -31.7 ± 1.7, k = 5 ± 0.5, n=15), R185H at 10°C (blue diamonds, $V_{1/2}$ = -23.8 ± 1.8, k = 6.7 ± 0.5, n=13). R185H was not significantly different when compared to WT ($p > 0.05$, Two-way ANOVA with Sidak's multiple comparison test, temperature and genotype are categorical variables). Currents elicited from a holding potential of -100 mV to different test pulse potentials (50ms) ranging from -80 to 40mV in 5mV increments. A holding potential of -100mV and an intersweep interval of 10s was used for all the protocols.

d) Steady-state fast inactivation curves for the WT at 20°C (black squares, $V_{1/2}$= -85.8 ± 1.8, k = 7.5 ± 0.6, n=14), WT at 10°C (grey dots, $V_{1/2}$ = -80 ± 1.8, k = 13.7 ± 0.6, n=13), R185H at 20°C (red triangles, $V_{1/2}$= -86.9 ± 1.5, k = 7.6 ± 0.3, n=14), R185H at 10°C (blue diamonds, $V_{1/2}$= -73.1 ± 2.4, k = 14.7 ± 0.6, n=13. R185H significantly different when compared to WT at 10°C ($p < 0.05$, Two-way ANOVA with Sidak's multiple comparison test, temperature and genotype are categorical variables). Currents elicited with test pulses to -10 mV following 500 ms inactivating prepulses.

e) Open-state fast-inactivation kinetics for the WT at 20°C (black squares, n=15), WT at 10°C (grey dots, n=14), R185H at 20°C (red triangles, n=15), R185H at 10°C (blue diamonds, n=13).

f) Normalized peak current-voltage relationship curves for the WT at 20°C (black squares, $V_{1/2}$ = -30.7 ± 1.3, k = 4.9 ± 0.4, n=15), WT at 30°C (light blue stars, $V_{1/2}$ = -34 ± 1, k = 5.2 ± 2.8, n=12), R185H at 20°C (red triangles, $V_{1/2}$ = -31.7 ± 1.7, k = 5 ± 0.5, n=15), R185H at 30°C (pink asterisks, $V_{1/2}$ = -33.6 ± 2, k = 4.8 ± 0.5, n=10). Currents elicited from a holding potential

of -100 mV to different test pulse potentials (50ms) ranging from -80 to 40mV in 5mV increments

g) Steady-state fast inactivation curves for the WT at 20°C (black squares, $V_{1/2}$ = -85.8 ± 1.8, k = 7.5 ± 0.6, n=14), WT at 30°C (light blue stars, $V_{1/2}$ = -86.3 ± 2.1, k = 7.5 ± 0.3, n=12), R185H at 20°C (red triangles, $V_{1/2}$ = -86.9 ± 1.5, k = 7.6 ± 0.3, n=14), R185H at 30°C (pink asterisks, $V_{1/2}$ = -85.9 ± 2.4, k = 9 ± 1.5, n=9). Currents elicited with test pulses to -10 mV following 500 ms inactivating prepulses.

h) Open-state fast-inactivation kinetics for WT at 20°C (black squares, n=15), WT at 30°C (light blue stars, n=12), ~~p.R185H~~p.Arg185His at 20°C (red triangles, n=15), R185H at 30°C (pink asterisks, n=10).

NFCI – Non-freezing cold injury
NPD – Neuropathic Pain Disorders
WT – Wild Type
Data are presented as mean ± SEM.
Statistical analysis for group comparisons - Two-way ANOVA with Sidak's multiple comparison test (*-statistically significant differences, P <0.05)

Figure 3

a) ~~p.A172V~~ p.Ala172Val variant: Alanine is substituted with a valine in the 4th ankyrin repeat domain of the channel. The residue is moderately conserved across different mammalian species.

b) Schematic of TRPA1 channel topology. ~~p.A172V~~p.Ala172Val variant is represented with a blue dot.

Voltage-dependence activation of hTRPA1 ~~p.A172V~~ p.Ala172Val and ~~p.N855S~~p.Asn855Ser was measured in response to AITC. Current-voltage curves were measured with a two voltage-step protocol (voltage-ramps ranging from -100 mV to + 100 mV for 500 ms, every 5 seconds). Holding potential was set at -70 mV. Application of 25µM of AITC shows enhanced activity of ~~p.A172V~~ p.Ala172Val variant in response to 25 µM AITC in the presence of extracellular calcium alone (c-g).

c) Current density, assessed using a two-voltage step protocol, was significantly different between WT and variant channels as quantified in d) and e)

d) Outward currents (+100mV, WT= 21.12 ± 2.46pA/pF, ~~p.A172V~~p.Arg185His= 56.92 ± 12.52pA/pF, ~~p.N855S~~p.Asn855Ser = 38.12 ± 10.77pA/pF) were significantly increased for ~~p.A172V~~ p.Ala172Val (p<0.005)

e) Inward currents (-100mV, WT= -6.12 ± 0.63pA/pF, ~~p.A172V~~p.Arg185His= -8.48 ± 1.37pA/pF, ~~p.N855S~~p.Asn855Ser = -13.48 ± 2.40pA/pF) were significantly increased for p.N885S (p<0.005)

f) Tail current analysis showed a significant shift in half-maximal activation potential for both ~~p.A172V~~ p.Ala172Val (n = 9, $V_{1/2}$ =35.55 ± 5.45mV, and ~~p.N855S~~p.Asn855Ser , $V_{1/2}$ = 37.03 ± 8.28mV n = 12, when compared to WT n = 13, $V_{1/2}$ = 59.16 ± 5.25mV; p<0.05). Slopes of the voltage-activation curve (i.e. voltage sensitivity) (k = 49.38 ± 2.03mV WT, k = 43.29 ± 2.63mV A172V, k = 45.20 ± 2.36mV; p=0.18) were not significantly different.

g) Current-voltage relationships were tested with a voltage-ramp protocol in which voltage changes at a steady rate and the resulting current is recorded. After administration of 25µM (arrow) averaged currents from voltage-ramps were extrapolated and at +90mV showed a three-fold increase in current densities at positive potentials after application of 25µM AITC (WT = 85.13 ± 23.30mV, ~~p.A172V~~p.Ala172Val = 250.4 ± 62.39mV, ~~p.N855S~~p.Asn855Ser = 256.1 ± 54.90mV; p<0.05). Insert shows example tracing.

WT – Wild Type
Data are presented as mean ± SEM.
Statistical analysis for group comparisons - One way ANOVA with Sidak's multiple comparison test (* - indicates statistically significant differences, P <0.05)

Figure 4:
Application of 100µM of Menthol in the presence of intracellular and extracellular calcium shows enhanced activity of ~~p.A172V~~p.Ala172Val and ~~p.N855S~~p.Asn855Ser variants. Current-voltage curves were measured with a two voltage-step protocol (voltage-ramps ranging from -100 mV to + 100 mV for 500 ms, every 5 seconds; holding potential was set at -70 mV).

a) Current density, assessed using a two-voltage step protocol, in WT and variant channels were significantly different as quantified in d) and e)
b) Outward currents were enhanced for both ~~p.A172V~~p.Ala172Val and ~~p.N855S~~p.Asn855Ser (+100mV, WT = 21.96 ± 3.64mV, p.Ala172Val = 39.94 ± 5.80mV, and ~~p.N855S~~p.Asn855Ser = 70.07 ± 21.71mV; p<0.05)
c) Inward currents were not statistically different (-100mV, WT = -8.29 ± 1.97mV, -6.52 ± 0.83mV p.Ala712Val, and ~~p.N855S~~p.Asn855Ser = -5.72 ± 0.94mV; p = 0.58, and p = 0.40, respectively)
d) Half-maximal activation potential was significantly shifted leftward for ~~p.A172V~~p.Ala172Val and ~~p.N855S~~p.Asn855Ser (V1/2, WT= 61.83 ± 7.37mV n = 8, ~~p.A172V~~p.Ala172Val = 36.41 ± 7.58mV n = 9, ~~p.N855S~~p.Asn855Ser = 33.60 ± 6.67mV n = 6). Voltage sensitivity was significantly altered (slope factor k = 41.51 ± 2.33mV WT; 40.21 ± 2.98mV ~~p.A172V~~p.Arg185His; 39.32 ± 3.03mV ~~p.N855S~~p.Asn855Ser ; p<0.05).

WT – Wild Type
Data are presented as mean ± SEM.
Statistical analysis for group comparisons - One way ANOVA with Sidak's multiple comparison test (* - indicates statistically significant differences, P <0.05)

Table 1

A summary of the neuropathic pain disorders and the key diagnostic criteria used to classify the study participants enrolled. Pattern of inherence for the Mendelian inherited pain disorders, genes (including the OMIM reference), and important PMID references and are listed.
NOS- Not otherwise specified
NPD- Neuropathic pain disorder.

Table 2

A summary of the genes shown to have a causal role in neuropathic pain and included in our analysis. At the time of recruitment, 2015, a list of 14 genes with an established causal role in neuropathic pain was curated (Tier 1 genes). The criteria for a gene inclusion were at least three independent families reported with a causal variant in the gene, or two families with additional in vitro functional studies and/or mouse model (Turro *et al.*, 2020).

If genes were implicated in neuropathic pain, but did not meet Tier 1 criteria, they were classified as Tier 2 genes. Genes of interest, as determined by expert consensus with little or no published data, were classified as Tier 3 genes. Before exploratory analysis of Tier 2 and 3 genes a contemporaneous search was performed to identify new genes that had been described in the ensuing period from the start of the project to the analysis of data (2015-2021). These genes were included in the tier 2 and 3 gene analysis

HSAN - Hereditary and Autonomic Sensory Neuropathy
HGNC - HUGO Gene Nomenclature Committee~~:~~

Table 3

List of gene variants that were reported ~~that~~ and were deemed medically actionable i.e. variants that result in specific, defined medical recommendations. Clinically relevant, medically actionable, variants were reported to the referring clinician.

All participants were heterozygous for the relevant variants
All variants were predicted by Ensembl Variant Effect Predictor as missense variants
VUS – variant of uncertain significance
gnomAD - Genome Aggregation Database, open resource that aggregates and harmonises both exome and genome sequencing data; current status lists 125,748 exome sequences and 15,708 whole-genome sequences from unrelated individuals sequenced as part of various disease-specific and population genetic studies.
Allele Frequencies are shown in the Neuropathic Pain Disorders (NPD) cohort and in NIHR BioResource control cohorts.
WGS10K – NIHR Bioresources database with 13,037 whole genome sequences.
NPD- Neuropathic Pain Disorders
In silico analysis, SIFT and Polyphen, results shown.

SIFT score predicts whether an amino acid substitution affects protein function. The SIFT score ranges from 0.0 (deleterious) to 1.0 (tolerated).
The score can be interpreted as follows:
0.0 to 0.05 - Variants with scores in this range are considered deleterious. Variants with scores closer to 0.0 are more confidently predicted to be deleterious.
0.05 to 1.0 -Variants with scores in this range are predicted to be tolerated (benign). Variants with scores very close to 1.0 are more confidently predicted to be tolerated.

Table 4
Results from the gene-wise association test (using the gene-wise approach of the combined burden and variance-component test SKAT-O) for rare variants of Tier 1-3 genes and functionally validated variants of the *SCN9A, SCN10A, SCN11A* genes for all participants with neuropathic pain.
Significance was set to the Bonferroni adjusted 0.01 threshold for the number of genes considered (0.01/36 = 2.7*10e-4). We report unadjusted P values that passed the Bonferroni corrected significant threshold.

The genomic coordinates and the HGNC gene symbol of the gene models that have a significantly different configuration of rare variants between cases and controls are presented in the first column. The second column holds the fraction of individuals carrying rare variants below the MAF threshold < 0.05. We the show the number of variants considered for the association, followed by the P value of the association test. The last column holds the Rho statistic.
Values can be interpreted as:
Rho = 0, causal and non-causal variants with opposing directions driving the association to Rho=1, high percentage of causal variants acting in the same direction driving the association.
Results are grouped by ethnicity and phenotype.

Supplementary table 1
Significant results from the gene-wise association test for rare variants of Tier 1-3 genes for each ethnicity and phenotype. Significance was set to the Bonferroni adjusted 0.01 threshold for the number of genes considered (0.01/36 = 2.7*10e-4). We report unadjusted P values that passed the Bonferroni corrected significant threshold.

Supplementary table 2
Significant results from the gene-wise association test for the whole gene set (5790). For the whole gene set analysis we applied a minor allele count filter of >= 2 and for the Neuropathic Pain Disorders candidate genes a minor allele count filter of >= 1. Only genes with at least two variants which passed the filters were included.
Significance was set to the Bonferroni adjusted 0.01 threshold for the number of genes considered (0.01/5790 = 1.7*10e-6).
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Supplementary figures and legends

Supplementary figure 1

Flow diagram outlining recruitment of participants, analysis pipeline and genetic testing and the difference between the clinical reporting of relevant gene variants versus the exploratory gene analysis and in vitro functional assessment of variants of interest

Supplementary figure 2

Flow diagram outlining neuropathic pain grading .
Possible neuropathic pain must fulfil criteria 1 and 2.
33tp3Probable neuropathic pain must fulfil criteria 1, 2 and 3.
Definite neuropathic pain must fulfil all 4 criteria.

1. Pain with a distinct neuroanatomically plausible distribution e.g. pain symmetrically distributed in the extremities, pain in erythematous areas
2. A history suggestive of a relevant lesion or disease affecting the peripheral or central somatosensory system e.g. a history of neuropathy symptoms including decreased sensation, or positive sensory symptoms e.g. burning, aching pain mainly in the toes, feet or legs.
3. Demonstration of distinct neuroanatomically plausible distribution of neuropathic pain e.g. presence of clinical signs of peripheral neuropathy such as decreased distal sensation or decreased/absent ankle reflexes.
4. Demonstration of the relevant lesion or disease by at least one confirmatory test e.g. abnormality on either nerve conduction tests, thermal thresholds or intra-epidermal nerve fibre density.

Supplementary figure 3

Biophysical characterisation of hTRPA1 WT, ~~p.A172V~~ p.Ala172Val and ~~p.N855S~~p.Asn855Ser variant in control conditions shows no significant differences. Voltage step protocol consisted of 400 ms voltage steps to test potentials ranging from –100 mV to +180 mV, followed by a final invariant step to -75 mV (400 ms to measure tail currents). The holding potential was set at -0 mV.

a) Current density measured in WT and mutant channels were not statistically different as quantified in b) and c)
b) Outward currents at (+100mV, WT = 15.41 ± 3.01pA/pF, ~~p.A172V~~ p.Ala172Val = 16.69 ± 2.85pA/pF, p. As~~N~~n855Ser = 23.81 ± 4.25pA/pF) were not significantly different between WT and the respective variants (p = 0.77 WT versus p.Ala172~~1~~V~~2~~al, p = 0.17 WT versus p.As~~N~~n855Ser)
c) Inward currents (at -100mV, WT = -5.42 ± 0.68pA/pF, p.Ala172Val = -6.80 ± 1.18pA/pF and ~~N855S~~p.Asn855Ser = -10.66 ± 3.00pA/pF), were not significantly different between WT and the respective variants (p = 0.75 WT versus p.Ala172~~71~~Val~~2~~, and p = 0.05 WT versus ~~N855S~~p.Asn855Ser).
d) Voltage of half-maximal activation for WT n = 12, ~~p.A172V~~ p.Ala172Val n = 9 and ~~p.N855S~~p.Asn855Ser n = 7 ($V_{1/2}$, WT = 59.70 ± 4.59mV, ~~p.A172V~~ p.Ala172Val = 59.45 ±

8.91mV, ~~p.N855S~~p.Asn855Ser = 64.85 ± 8.09mV; p= 0.99, and p = 0.85, respectively) and slopes of the voltage-activation curve (k, WT = 42.51 ± 2.01mV, ~~p.A172V~~ p.Ala172Val = 46.19 ± 2.60mV, ~~p.N855S~~p.Asn855Ser = 47.45 ± 1.88mV; p = 0.41 and p = 0.26, respectively) were not statistically different.

WT – Wild Type
Data are presented as mean ± SEM.
Statistical analysis for group comparisons - One way ANOVA with Sidak's multiple comparison test (* - indicates statistically significant differences, P <0.05)

Supplementary figure 4:
Application of 100μM of Menthol, with extracellular calcium alone, did not alter ~~p.A172V~~ p.Ala172Val nor ~~p.N855S~~p.Asn855Ser channel excitability. Voltage-dependence activation of hTRPA1 ~~p.A172V~~ p.Ala172Val and ~~p.N855S~~p.Asn855Ser was measured in response to Menthol. Current-voltage curves were measured with a two voltage-step protocol (voltage-ramps ranging from -100 mV to + 100 mV for 500 ms, every 5 seconds; holding potential was set at -70 mV).

a) Current densities were not statistically different for the variant channels when compared to WT as quantified in b) and c)
b) Outward current (at +100mV, WT = 13.29 ± 1.69pA/pF, ~~p.A172V~~ p.Ala172Val = 17.51 ± 2.60pA/pF, ~~p.N855S~~p.Asn855Ser = 20.66 ± 3.35pA/pF were not significantly different (p = 0.44, and p = 0.15, respectively).
c) Inward currents (at -100mV, WT = -6.02 ± 1.11pA/pF, ~~p.A172V~~ p.Ala172Val = -7.66 ± 1.83pA/pF, ~~p.N855S~~p.Asn855Ser = -7.49 ± 1.53pA/pF) were not significantly different (p = 0.72, p = 0.80, respectively).
d) Half-maximal activation voltage for WT n = 8, ~~p.A172V~~ p.Ala172Val n = 8, and ~~N855S~~p.Asn855Ser n = 7 ($V_{1/2}$, WT= 65.39 ± 5.79mV, ~~p.A172V~~p.Arg185His= 56.61 ± 9.25mV, p.Asn855Ser~~p. N855S~~p.Asn855Ser = 58.62 ± 7.31mV, p = 0.69, and p = 0.83, respectively) were not statistically different. Slopes of the voltage-activation curve (k = 56.66 ± 1.40mV WT, 49.01 ± 1.53mV ~~p.A172V~~p.Arg185His, 49.73 ± 3.28mV ~~N855S~~p.Asn855Ser, p= 0.022 and p = 0.069, respectively) were reduced for both p.A712V and ~~p.N855S~~p.Asn855Ser .

WT – Wild Type
Data are presented as mean ± SEM.
Statistical analysis for group comparisons - One way ANOVA with Sidak's multiple comparison test (* - indicates statistically significant differences, P <0.05).

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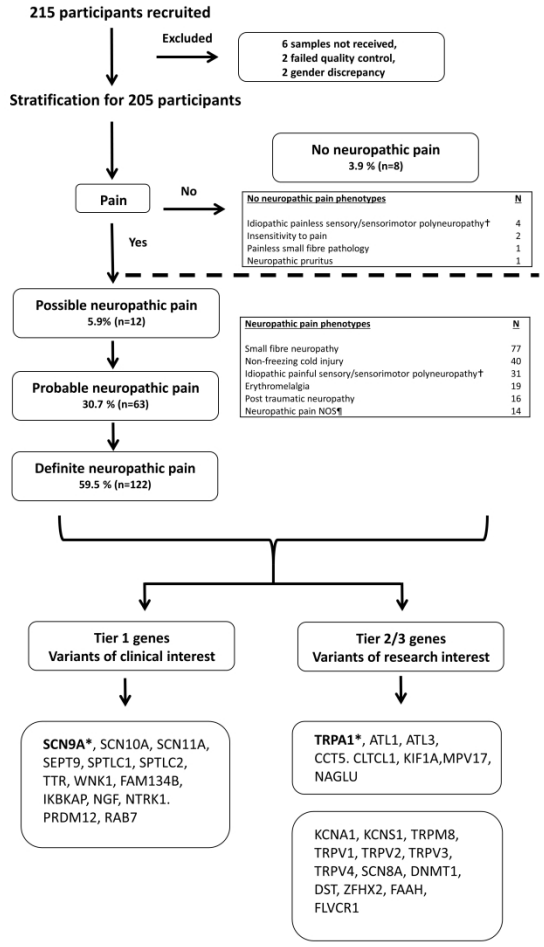

Figure 1

Figure 1
Flow diagram outlining neuropathic pain grading, study participant recruitment and summary of study participant clinical phenotype
Ten participants were excluded due to either samples not received, quality control failures or gender discrepancies.
□☐- Included cases of longstanding progressive neuropathies where the presenting or predominant feature was altered sensory function and an underlying cause could not be identified
¶ - Include cases of post herpetic neuralgia, episodic pain syndromes, neuropathic pain with plausible neuroanatomical distribution but no abnormalities on examination and specialised tests, leprosy, hereditary neuralgic amyotrophy, type 1 complex regional pain syndrome, Noonan syndrome, injury to left arm (these cases were included due to severe pain which was in excess of the inciting injury).
* - Genes selected for electrophysiological characterisation
NOS- Not otherwise specified

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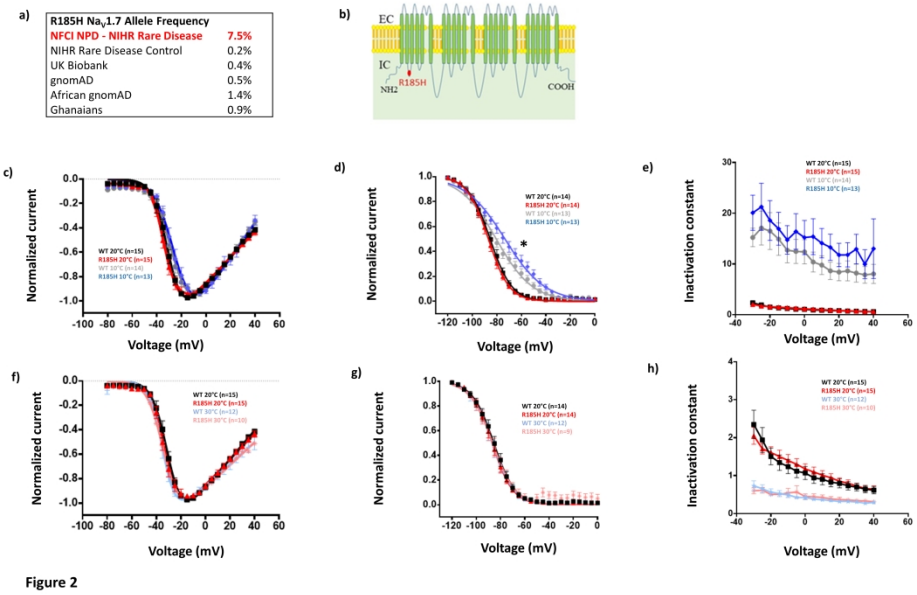

Figure 2

Figure 2

a) p.R185H is more common in non-freezing cold injury than control populations.

b) Schematic of NaV1.7 channel topology. R185H is represented with a red dot.

c) Normalized peak current-voltage relationship curves for the WT at 20°C (black squares, V1/2 = -30.7 ± 1.3, k = 4.9 ± 0.4, n=15), WT at 10°C (grey dots, V1/2 = -26.5 ± 2, k = 6.6 ± 0.6, n=14), R185H at 20°C (red triangles, V1/2 = -31.7 ± 1.7, k = 5 ± 0.5, n=15), R185H at 10°C (blue diamonds, V1/2 = -23.8 ± 1.8, k = 6.7 ± 0.5, n=13). R185H was not significantly different when compared to WT ($p > 0.05$). Currents elicited from a holding potential of -100 mV to different test pulse potentials (50ms) ranging from -80 to 40mV in 5mV increments. A holding potential of -100mV and an intersweep interval of 10s was used for all the protocols.

d) Steady-state fast inactivation curves for the WT at 20°C (black squares, V1/2= -85.8 ± 1.8, k = 7.5 ± 0.6, n=14), WT at 10°C (grey dots, V1/2 = -80 ± 1.8, k = 13.7 ± 0.6, n=13), R185H at 20°C (red triangles, V1/2= -86.9 ± 1.5, k = 7.6 ± 0.3, n=14), R185H at 10°C (blue diamonds, V1/2= -73.1 ± 2.4, k = 14.7 ± 0.6, n=13. R185H significantly different when compared to WT at 10°C ($p < 0.05$). Currents elicited with test pulses to -10 mV following 500 ms inactivating prepulses.

e) Open-state fast-inactivation kinetics for the WT at 20°C (black squares, n=15), WT at 10°C (grey dots, n=14), R185H at 20°C (red triangles, n=15), R185H at 10°C (blue diamonds, n=13).

f) Normalized peak current-voltage relationship curves for the WT at 20°C (black squares, V1/2 = -30.7 ± 1.3, k = 4.9 ± 0.4, n=15), WT at 30°C (light blue stars, V1/2 = -34 ± 1, k = 5.2 ± 2.8, n=12), R185H at 20°C (red triangles, V1/2 = -31.7 ± 1.7, k = 5 ± 0.5, n=15), R185H at 30°C (pink asterisks, V1/2 = -33.6 ± 2, k = 4.8 ± 0.5, n=10). Currents elicited from a holding potential of -100 mV to different test pulse potentials (50ms) ranging from -80 to 40mV in 5mV increments

g) Steady-state fast inactivation curves for the WT at 20°C (black squares, V1/2 = -85.8 ± 1.8, k = 7.5 ± 0.6, n=14), WT at 30°C (light blue stars, V1/2 = -86.3 ± 2.1, k = 7.5 ± 0.3, n=12), R185H at 20°C (red triangles, V1/2 = -86.9 ± 1.5, k = 7.6 ± 0.3, n=14), R185H at 30°C (pink asterisks, V1/2 = -85.9 ± 2.4, k = 9 ± 1.5, n=9). Currents elicited with test pulses to -10 mV following 500 ms inactivating prepulses.

h) Open-state fast-inactivation kinetics for WT at 20°C (black squares, n=15), WT at 30°C (light blue stars, n=12), p.R185H at 20°C (red triangles, n=15), R185H at 30°C (pink asterisks, n=10).

Data are presented as mean ± SEM.

Statistical analysis for group comparisons - Two-way ANOVA with Sidak's multiple comparison test, temperature and genotype are categorical variables (*-statistically significant differences, $P < 0.05$)

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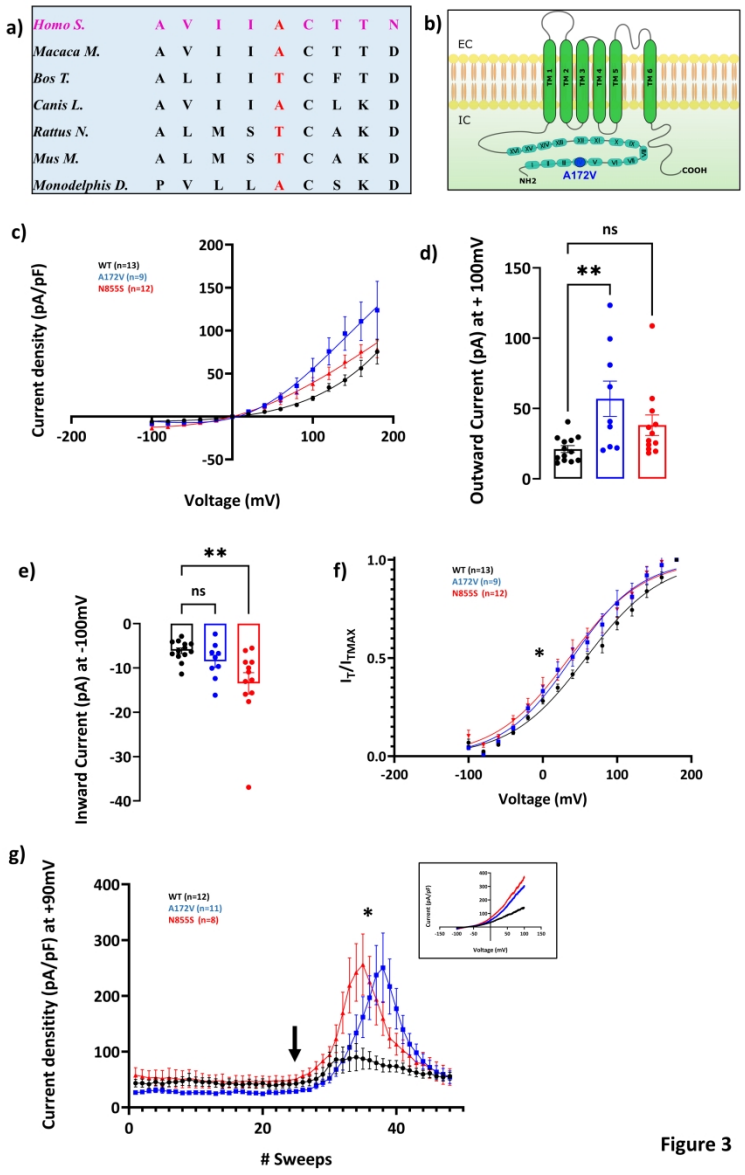

Figure 3

Figure 3

a) p.A172V variant: Alanine is substituted with a valine in the 4th ankyrin repeat domain of the channel. The residue is moderately conserved across different mammalian species.

b) Schematic of TRPA1 channel topology. p.A172Vvariant is represented with a blue dot.

Voltage-dependence activation of hTRPA1 p.A172V and p.N855S was measured in response to AITC. Current-voltage curves were measured with a two voltage-step protocol (voltage-ramps ranging from -100 mV to + 100 mV for 500 ms, every 5 seconds). Holding potential was set at -70 mV. Application of 25μM of AITC shows enhanced activity of p.A172Vvariant in response to 25 μM AITC in the presence of extracellular calcium alone (c-g).

c) Current density, assessed using a two-voltage step protocol, was significantly different between WT and variant channels as quantified in d) and e)

d) Outward currents (+100mV, WT= 21.12 ± 2.46pA/pF, p.A172V= 56.92 ± 12.52pA/pF, p.N855S = 38.12 ± 10.77pA/pF) were significantly increased for p.A172V ($p<0.005$)

e) Inward currents (-100mV, WT= -6.12 ± 0.63pA/pF, p.A172V= -8.48 ± 1.37pA/pF, p.N855S = -13.48 ± 2.40pA/pF) were significantly increased for p.N885S ($p<0.005$)
f) Tail current analysis showed a significant shift in half-maximal activation potential for both p.A172V (n = 9, V1/2 =35.55 ± 5.45mV, and p.N855S, V1/2 = 37.03 ± 8.28mV n = 12, when compared to WT n = 13, V1/2 = 59.16 ± 5.25mV; $p<0.05$). Slopes of the voltage-activation curve (i.e. voltage sensitivity) (k = 49.38 ± 2.03mV WT, k = 43.29 ± 2.63mV A172V, k = 45.20 ± 2.36mV; $p=0.18$) were not significantly different.
g) Current-voltage relationships were tested with a voltage-ramp protocol in which voltage changes at a steady rate and the resulting current is recorded. After administration of 25μM (arrow) averaged currents from voltage-ramps were extrapolated and at +90mV showed a three-fold increase in current densities at positive potentials after application of 25μM AITC (WT = 85.13 ± 23.30mV, p.A172V = 250.4 ± 62.39mV, p.N855S = 256.1 ± 54.90mV; $p<0.05$). Insert shows example tracing.

WT – Wild Type
Data are presented as mean ± SEM.
Statistical analysis for group comparisons - One way ANOVA with Sidak's multiple comparison test (* - indicates statistically significant differences, $P <0.05$)

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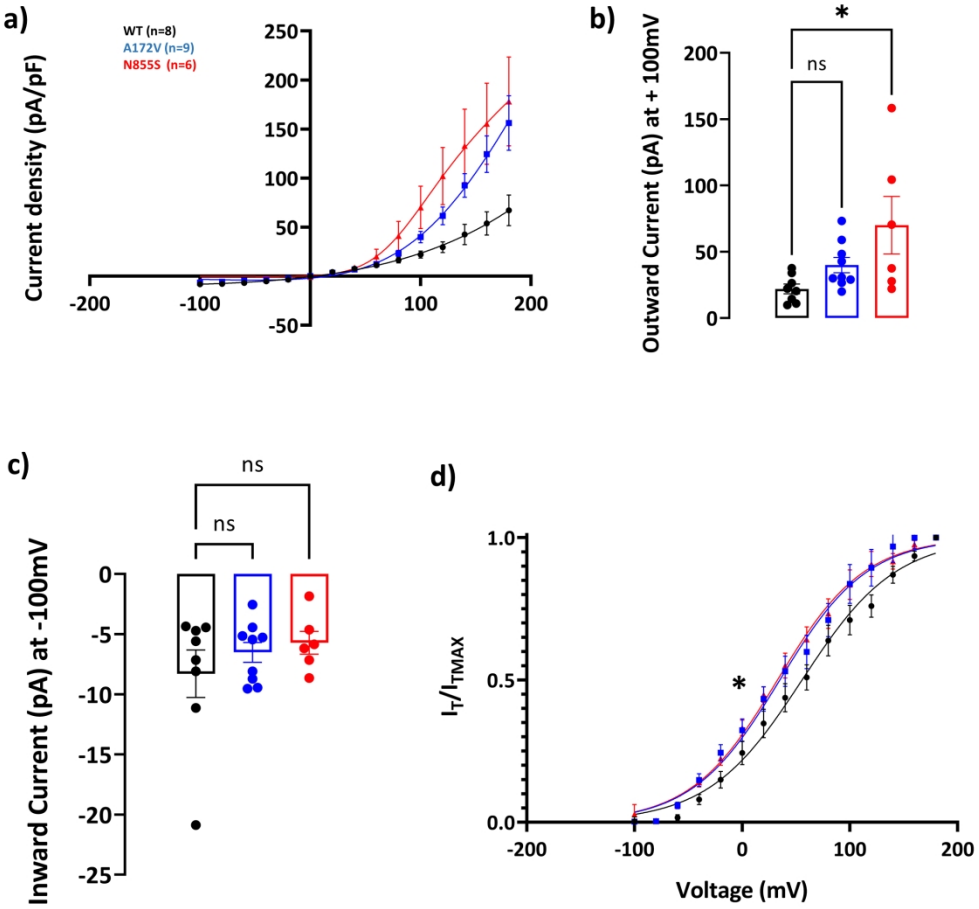

Figure 4

Figure 4:
Application of 100μM of Menthol in the presence of intracellular and extracellular calcium shows enhanced activity of p.A172V and p.N855S variants. Current-voltage curves were measured with a two voltage-step protocol (voltage-ramps ranging from -100 mV to + 100 mV for 500 ms, every 5 seconds; holding potential was set at -70 mV).

a) Current density, assessed using a two-voltage step protocol, in WT and variant channels were significantly different as quantified in d) and e)
b) Outward currents were enhanced for both p.A172V and p.N855S (+100mV, WT = 21.96 ± 3.64mV, p.A172 = 39.94 ± 5.80mV, and p.N855S = 70.07 ± 21.71mV; $p<0.05$)
c) Inward currents were not statistically different (-100mV, WT = -8.29 ± 1.97mV, -6.52 ± 0.83mV p.A712V, and p.N855S = -5.72 ± 0.94mV; $p = 0.58$, and $p = 0.40$, respectively)
d) Half-maximal activation potential was significantly shifted leftward for p.A172V and p.N855S (V1/2, WT= 61.83 ± 7.37mV n = 8, p.A172V = 36.41 ± 7.58mV n = 9, p.N855S = 33.60 ± 6.67mV n = 6). Voltage sensitivity was significantly altered (slope factor k = 41.51 ± 2.33mV WT; 40.21 ± 2.98mV p.A172V; 39.32 ± 3.03mV p.N855S; $p<0.05$).

WT – Wild Type
Data are presented as mean ± SEM.
Statistical analysis for group comparisons - One way ANOVA with Sidak's multiple comparison test (* - indicates statistically significant differences, $P <0.05$)

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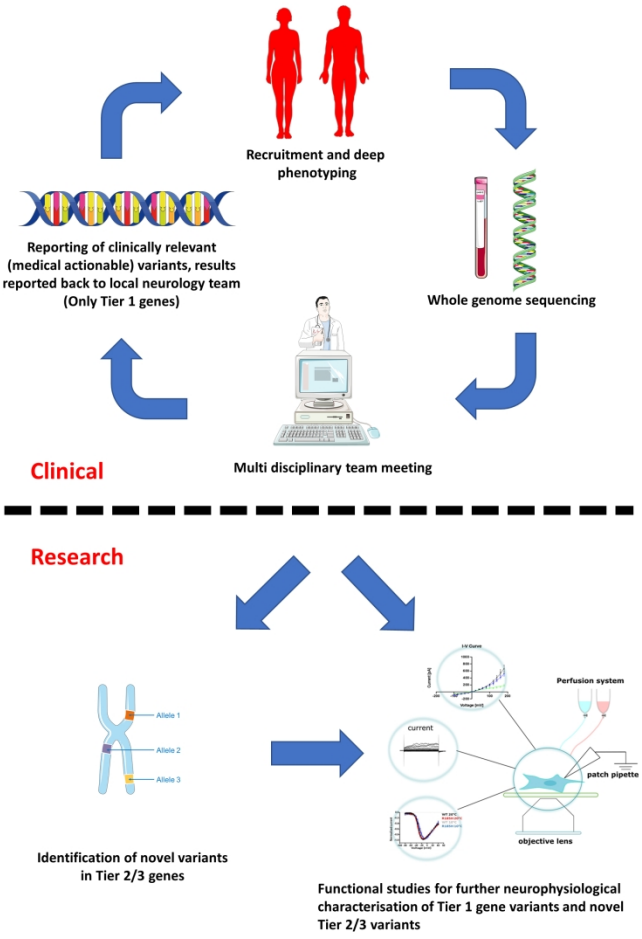

Supplementary Figure 1

Supplementary figure 1
Flow diagram outlining recruitment of participants, analysis pipeline and genetic testing and the difference between the clinical reporting of relevant gene variants versus the exploratory gene analysis and in vitro functional assessment of variants of interest

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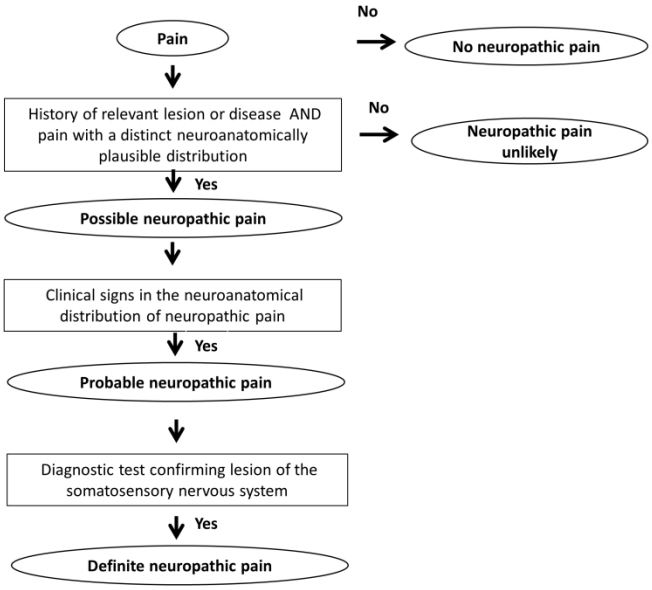

Supplementary Figure 2

Supplementary figure 2
Flow diagram outlining neuropathic pain grading .
Possible neuropathic pain must fulfil criteria 1 and 2.
Probable neuropathic pain must fulfil criteria 1, 2 and 3.
Definite neuropathic pain must fulfil all 4 criteria.

1. Pain with a distinct neuroanatomically plausible distribution e.g. pain symmetrically distributed in the extremities, pain in erythematous areas
2. A history suggestive of a relevant lesion or disease affecting the peripheral or central somatosensory system e.g. a history of neuropathy symptoms including decreased sensation, or positive sensory symptoms e.g. burning, aching pain mainly in the toes, feet or legs.
3. Demonstration of distinct neuroanatomically plausible distribution of neuropathic pain e.g. presence of clinical signs of peripheral neuropathy such as decreased distal sensation or decreased/absent ankle reflexes.

4. Demonstration of the relevant lesion or disease by at least one confirmatory test e.g. abnormality on either nerve conduction tests, thermal thresholds or intra-epidermal nerve fibre density.

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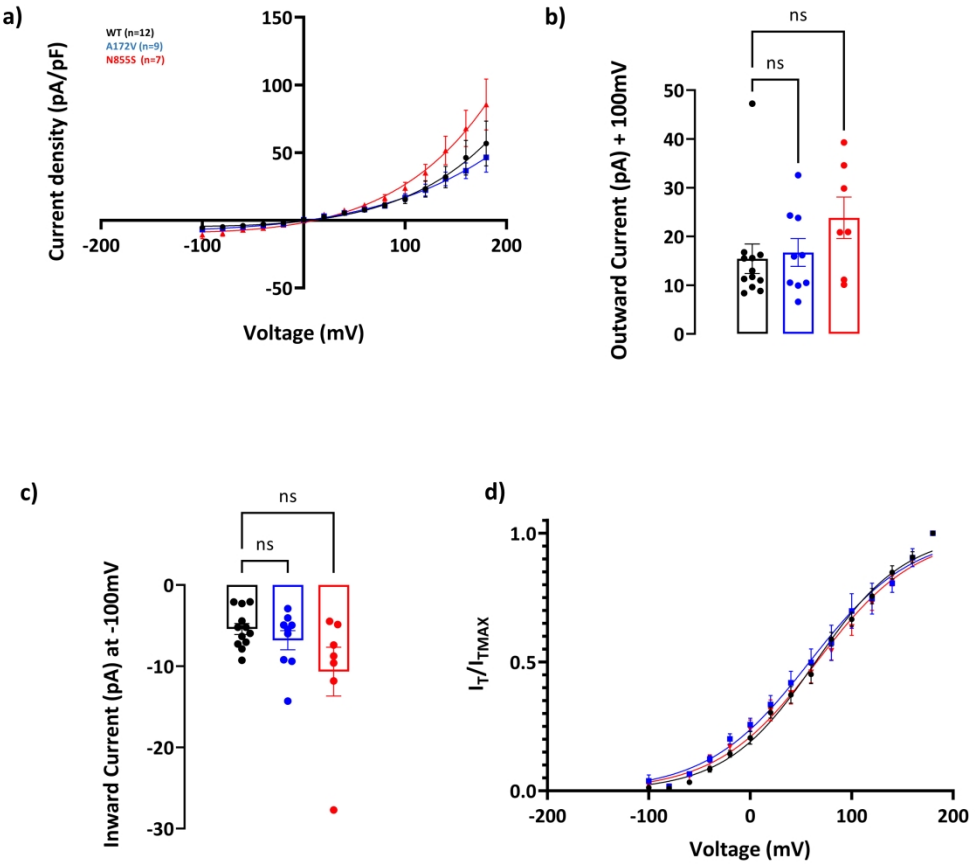

Supplementary Figure 3

Supplementary figure 3
Biophysical characterisation of hTRPA1 WT, p.A172V and p.N855S variant in control conditions shows no significant differences. Voltage step protocol consisted of 400 ms voltage steps to test potentials ranging from −100 mV to +180 mV, followed by a final invariant step to -75 mV (400 ms to measure tail currents). The holding potential was set at -0 mV.

a) Current density measured in WT and mutant channels were not statistically different as quantified in b) and c)
b) Outward currents at (+100mV, WT = 15.41 ± 3.01pA/pF, p.A172V = 16.69 ± 2.85pA/pF, N855S = 23.81 ± 4.25pA/pF) were not significantly different between WT and the respective variants (p = 0.77 WT versus A71V2, p = 0.17 WT versus N855S)
c) Inward currents (at -100mV, WT = -5.42 ± 0.68pA/pF, A172V = -6.80 ± 1.18pA/pF and N855S = -10.66 ± 3.00pA/pF), were not significantly different between WT and the respective variants (p = 0.75 WT versus A71V2, and p = 0.05 WT versus N855S).
d) Voltage of half-maximal activation for WT n = 12, p.A172V n = 9 and p.N855S n = 7 (V1/2, WT = 59.70 ± 4.59mV, p.A172V = 59.45 ± 8.91mV, p.N855S = 64.85 ± 8.09mV; p= 0.99, and p = 0.85, respectively) and slopes of the voltage-activation curve (k, WT = 42.51 ± 2.01mV, p.A172V = 46.19 ± 2.60mV, p.N855S = 47.45 ± 1.88mV; p = 0.41 and p = 0.26, respectively) were not statistically different.

WT – Wild Type
Data are presented as mean ± SEM.

Statistical analysis for group comparisons - One way ANOVA with Sidak's multiple comparison test (* - indicates statistically significant differences, P <0.05)

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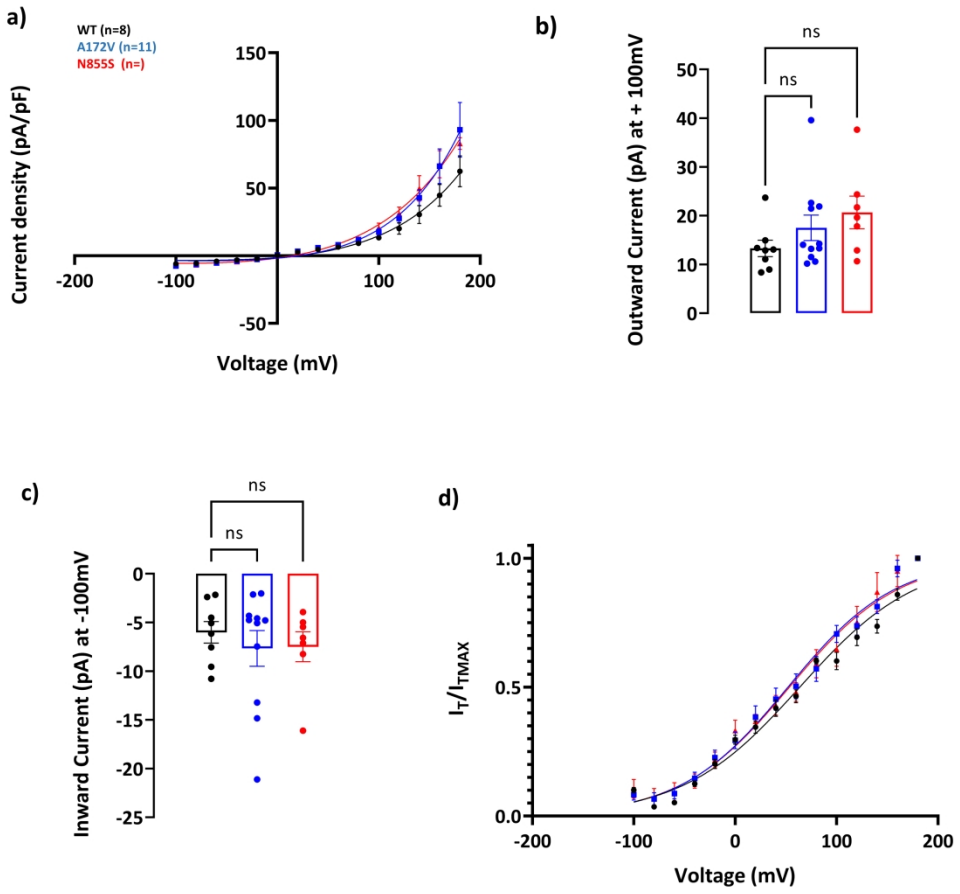

Supplementary Figure 4

Supplementary figure 4:
Application of 100μM of Menthol, with extracellular calcium alone, did not alter p.A172V nor p.N855S channel excitability. Voltage-dependence activation of hTRPA1 p.A172V and p.N855S was measured in response to Menthol. Current-voltage curves were measured with a two voltage-step protocol (voltage-ramps ranging from -100 mV to + 100 mV for 500 ms, every 5 seconds; holding potential was set at -70 mV).

a) Current densities were not statistically different for the variant channels when compared to WT as quantified in b) and c)
b) Outward current (at +100mV, WT = 13.29 ± 1.69pA/pF, p.A172V = 17.51 ± 2.60pA/pF, p.N855S = 20.66 ± 3.35pA/pF were not significantly different (p = 0.44, and p = 0.15, respectively).
c) Inward currents (at -100mV, WT = -6.02 ± 1.11pA/pF, p.A172V = -7.66 ± 1.83pA/pF, p.N855S = -7.49 ± 1.53pA/pF) were not significantly different (p = 0.72, p = 0.80, respectively).
d) Half-maximal activation voltage for WT n = 8, p.A172V n = 8, and N855S n = 7 (V1/2, WT= 65.39 ± 5.79mV, p.A172V= 56.61 ± 9.25mV, p. N855S = 58.62 ± 7.31mV, p = 0.69, and p = 0.83, respectively) were not statistically different. Slopes of the voltage-activation curve (k = 56.66 ± 1.40mV WT, 49.01 ± 1.53mV p.A172V, 49.73 ± 3.28mV N855S, p= 0.022 and p = 0.069, respectively) were reduced for both p.A712V and p.N855S.

WT – Wild Type
Data are presented as mean ± SEM.

Statistical analysis for group comparisons - One way ANOVA with Sidak's multiple comparison test (* - indicates statistically significant differences, P <0.05).

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Table 1

NPD	Diagnostic criteria	Associated phenotypes	Likely inheritance	Known gene (OMIM #)	PMID reference
Congenital insensitivity to pain (including Hereditary sensory and autonomic neuropathy type IV, V, VII)	Inability to perceive painful stimuli Other somatosensory modalities may be impaired but the predominant clinical presentation is loss of pain sensibility	Anosmia Autonomic dysfunction Anhydrosis Intellectual impairment	AD, AR	SCN9A (243000), NGF (608654), NTRK1 (256800), SCN11A (615548), PRDM12 (616488), MPV17 (256810), CLTCL1 (601273)	17167479, 17470132, 17597096, 23596073, 19304393, 14976160, 8696348, 24036948, 26005867, 185990, 26068709
Hereditary sensory and autonomic neuropathy type I, II, III	Progressive neuropathies where the presenting or predominant feature is altered sensory function	Autonomic features and motor nerve involvement	AD, AR, X-linked	SPLTLC1 (162400), SPTLC2 (605713), WNK1 (201300), RAB7 (600882), IKBKAP (223900), FAM134B (613115), KIF1A (614213), ATL1 (613708), ATL3 (615632), CCT5 (256840)	11242114, 20920666, 15060842, 12545426, 8102296, 19838196, 21820098, 21194679, 24459106, 16399879
Erythromelalgia	Pain and erythema of the extremities which is exacerbated by warming and relieved by cooling. Initially episodic but may become persistent.	Onset by age 20	AD	SCN9A (133020)	14985375
Familial episodic pain syndrome	Severe episodic pain usually localised to the trunk and limbs with no structural cause. Triggers include cold environment, exercise and fasting.	Onset usually in childhood Possible family history	AD	TRPA1 (615040) , SCN11A (615552)	20547126, 24207120
Small fibre neuropathy	Probable—symptoms in hands and feet consistent with small fibre dysfunction (pain and altered temperature sensibility), clinical signs of small fibre damage (reduced pin prick sensitivity and ability to discriminate warm/cool) and normal nerve conduction studies. Definite—symptoms in hands and feet, clinical signs of small fibre damage, normal nerve conduction studies, and altered intra-epidermal nerve fibre density at the ankle and/or abnormal quantitative sensory testing of thermal thresholds at the foot	Autonomic features	AD	SCN9A (133020), SCN10A (615551), SCN11A (615552)	21698661, 23115331, 24207120
Post traumatic neuropathy	Traumatic nerve injury with clinical evidence of nerve injury in the neuroanatomical distribution of neuropathic pain		N/A	N/A	
Neuropathic pain NOS	Pain with a distinct neuroanatomically plausible distribution; however, no evidence of nerve injury found on clinical examination or specialised investigations		N/A	N/A	

Table 1

Table 2

Gene symbol	HGNC database: ID	Chromosomal position	HGNC database: description	Clinical phenotypes	OMIM	PMID Reference
Tier 1 genes						
FAM134B	25964	5p15.1	family with sequence similarity 134 member B	HSAN 2B;	613115	19838196, 21115472, 24327336
IKBKAP	5959	9q31	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein	Riley Day Syndrome/HSAN 3/Familial Dysautonomia	223900	8102296, 11179021, 11179008
NGF	7808	1p13.1	nerve growth factor	HSAN 5	608654	14976160, 20978020
NTRK1	8031	1q21-q22	neurotrophic receptor tyrosine kinase 1	HSAN 4/Congenital insensitivity to pain with anhidrosis	256800	8696348, 11668614, 18077166
PRDM12	13997	9q34.12	PR domain 12	HSAN 8/Congenital insensitivity to pain	616488	26005867, 26975306
RAB7	9788	3q21	RAB7A, member RAS oncogene family	HSAN1/2B	600882	12545426,
SCN9A	10597	2q24.3	sodium voltage-gated channel alpha subunit 9	Congenital insensitivity to pain, primary erythromelalgia, paroxysmal extreme pain disorder	243000, 133020	17167479, 14985375, 17145499
SCN10A	10582	3p22.2	sodium voltage-gated channel alpha subunit 10	Painful small fibre neuropathy	615551	23115331, 24006052, 26711856
SCN11A	10583	3p22.2	sodium voltage-gated channel alpha subunit 11	Familial episodic pain; Insensitivity to pain	615552, 615548	24036948, 24207120, 24776970
SEPT9	7323	17q25.3	septin 9	Hereditary neuralgic amyotrophy	162100	16186812, 19451530, 21556032
SPTLC1	11277	9q22.31	serine palmitoyltransferase long chain base subunit 1	HSAN 1	162400	11242114,11242106, 15037712,
SPTLC2	11278	14q24.3	serine palmitoyltransferase long chain base subunit 2	HSAN 1	605713	12207934, 20920666, 23658386
TTR	12405	18q12.1	transthyretin	Familial amyloidosis	105210	3011930, 14640030
WNK1	14540	12p13.3	WNK lysine deficient protein kinase 1	HSAN 2	201300	15060842, 18521183, 21625937
Tier 2 genes						
ATL1	HGNC:11231	14q21.3	atlastin GTPase 1	HSAN 1; Hereditary Spastic Paraplegia	613708	21194679, 22340599
ATL3	HGNC:24526	11q13.1	atlastin GTPase 3	HSAN 1	615632	24459106, 30680846
CCT5	HGNC:1618	5p15.2	chaperonin containing TCP1 subunit 5	HSAN with spastic paraplegia	256840	16399879
CLTCL1	HGNC:2093	22q11.2	clathrin heavy chain like 1	HSAN 5/Congenital insensitivity to pain	601273	26068709
KIF1A	HGNC:888	2q37.2	kinesin family member 1A	HSAN 2; Hereditary Spastic Paraplegia	614213	21820098, 25265257

MPV17	HGNC:7224	2p23.3	MPV17, mitochondrial inner membrane protein	Congenital neuropathy leads to absent pain at birth in severe cases. Hepatic failure and encephalopathy overshadow the neuropathy.	256810	185990, 11431741
NAGLU	HGNC:7632	17q21.2	N-acetyl-alpha-glucosaminidase	Painful axonal polyneuropathy in heterozygotes; mucopolysacharidosis IIIB when homozygous		25818867
TRPA1	HGNC:497	8q13	transient receptor potential cation channel subfamily A member 1	Familial episodic pain syndrome	615040	20547126,16564016
Tier 3 genes						
KCNA1	HGNC:6218	12p13	potassium voltage-gated channel subfamily A member 1			20724292
KCNS1	HGNC:6300	20q12	potassium voltage-gated channel modifier subfamily S member 1	No specific Mendelian disorder but the rs734784 allele enhances risk of nerve pain following nerve injury		
TRPM8	HGNC:17961	2q37	transient receptor potential cation channel subfamily M member 8			
TRPV1	HGNC:12716	17p13.2	transient receptor potential cation channel subfamily V member 1			9349813, 9768840
TRPV2	HGNC:18082	17p11.2	transient receptor potential cation channel subfamily V member 2			
TRPV3	HGNC:18084	17p13.3	transient receptor potential cation channel subfamily V member 3			
TRPV4	HGNC:18083	12q24.11	transient receptor potential cation channel subfamily V member 4			
SCN8A	HGNC:10596	12q13.1	sodium voltage-gated channel alpha subunit 8			22493249
SETDB2	HGNC:20263	13q14	SET domain bifurcated 2	Mutation identified in this group: HSN/Insensitivity to pain (unpublished data DLB)		
Additional genes after contemporaneous search						
DNMT1	HGNC:2976	19p13.2	DNA methyltransferase 1	Neuropathy, hereditary sensory, type IE	126375	21532572, 23365052, 25678562
Dystonin	HGNC:1090	6p12.1	Cytoskeleton linker protein	hereditary sensory and autonomic neuropathy type VI (HSAN6)	614653	22522446
ZFHX2	HGNC:20152	14q11.2	zinc finger homeobox 2	Congenital insensitivity to pain Marsili syndrome (MARSIS)	243000	29253101
FAAH	HGNC:50679	1p33	fatty acid amide hydrolase pseudogene 1	Insensitivity to pain	243000	30929760
FLVCR1	HGNC:24682	1q32.3	FLVCR heme transporter 1	Sensory neurodegeneration with loss of pain perception	609144	27923065

Table 2

Table 3

No	Clinical phenotype	Neuropathic pain grading	Gene	Nucleotide change	Amino acid change	Assigned pathogenicity	Allele frequency of variants in databases				In silico analysis		Previous study demonstrating altered channel function (PMID Reference)
							gnomAD	WGS10K	NPD European (African)	NIHR Bioresources controls European (African)	SIFT score	Polyphen	
1	Erythromelalgia	Definite	SCN9A	c.2543T>C	p.Ile848Thr p.I848T	~~Clearly~~ pPathogenic	.	0.0001	0.0039 (0)	0 (0)	0.00	probably damaging	Yes (15385606)
2	Erythromelalgia	Definite	SCN9A	c.2543T>C	p.Ile848Thr p.I848T	~~Clearly~~ pPathogenic	.	0.0001	0.0039 (0)	0 (0)	0.00	probably damaging	Yes (15385606)
3	Sensorimotor neuropathy	Probable	SPTLC1	c.399T>G	p.Cys133Trpp.C133W	~~Clearly~~ pPathogenic	.	0.0000	0.0039 (0)	0 (0)	0.00	probably damaging	Yes (16210380)
4	Small fibre neuropathy	Definite	SCN10A	c.4984G>A	p.Gly1662Serp.G1662S	Likely pathogenic	0.0014	0.0010	0.0039 (0)	0.0005 (0)	0.00	probably damaging	Yes (23115331)
5	Small fibre neuropathy	Definite	SCN11A	c.4628G>A	p.Cys1543Tyrp.C1543Y	Likely pathogenic	0.0001	0.00004	0.0039 (0.0131)	0 (0)	0.01	probably damaging	No studies for this variant
6	Painful sensory neuropathy	Definite	SPTLC2	c.886A>C	p.Ile296Leu p.I296L	VUS	.	0.0000	0.0039 (0)	0 (0)	0.04	possibly damaging	No studies for this variant
7	Non-freezing cold injury	Definite	SCN9A	c.554G>A	p.R185H p.Arg185His	VUS	0.0031	0.0016	0 (0.0921)	0.0015 (0.0064)	0.01	probably damaging	Yes (21698661, 22826602)
8	Non-freezing cold injury	Definite	SCN9A	c.554G>A	p.Arg185His p.R185H	VUS	0.0031	0.0016	0 (0.0921)	0.0015 (0.0064)	0.01	probably damaging	Yes (21698661, 22826602)
9	Non-freezing cold injury	Definite	SCN9A	c.554G>A	p.Arg185His p.R185H	VUS	0.0031	0.0016	0 (0.0921)	0.0015 (0.0064)	0.01	probably damaging	Yes (21698661, 22826602)
10	Non-freezing cold injury	Definite	SCN9A	c.554G>A	p.Arg185His p.R185H	VUS	0.0031	0.0016	0 (0.0921)	0.0015 (0.0064)	0.01	probably damaging	Yes (21698661, 22826602)
11	Non-freezing cold injury	Definite	SCN9A	c.554G>A	p.Arg185His p.R185H	VUS	0.0031	0.0016	0 (0.0921)	0.0015 (0.0064)	0.01	probably damaging	Yes (21698661, 22826602)
12	Non-freezing cold injury	Definite	SCN9A	c.554G>A	p.Arg185His p.R185H	VUS	0.0031	0.0016	0 (0.0921)	0.0015 (0.0064)	0.01	probably damaging	Yes (21698661, 22826602)
13	Small fibre neuropathy	Definite	SCN9A	c.4612T>C	p.Trp1538Argp.W1538R	VUS	0.0020	0.0018	0.0039 (0)	0.0018 (0)	0.71	benign	Yes (23292638)
14	Small fibre neuropathy	Definite	SCN9A	c.1445A>G	p.K482Rp.Lys482Arg	VUS	0	0.00004	0.0039 (0)	0 (0)	0.18	benign	No studies for this variant
15	Small fibre neuropathy	Probable	SCN9A	c.2215A>G	p.Ile739Val p.I739V	VUS	0.0024	0.0032	0.0078 (0)	0.0041 (0)	0.03	benign	Yes (22826602)
16	Small fibre neuropathy	Definite	SCN9A	c.2215A>G	p.Ile739Val p.I739V	VUS	0.0024	0.0032	0.0078 (0)	0.0041 (0)	0.03	benign	Yes (22826602)
17	Painful sensory neuropathy	Probable	SCN9A	c.2215A>G	p.Ile739Val p.I739V	VUS	0.0024	0.0032	0.0078 (0)	0.0041 (0)	0.03	benign	Yes (22826602)
18	Sensorimotor neuropathy	Definite	SCN9A	c.2215A>G	p.Ile739Val p.I739V	VUS	0.0024	0.0032	0.0078 (0)	0.0041 (0)	0.03	benign	Yes (22826602)
19	Small fibre neuropathy	Definite	SCN9A	c.4982A>G	p.Glu1661Glyp.E1661G	VUS	0.0000	0.0001	0.0039 (0)	0.0018 (0)	0.10	benign	No studies for this variant
20	Traumatic neuropathy	Probable	SCN9A	c.554G>A	p.Arg185His p.R185H	VUS	0.0031	0.0016	0 (0.0921)	0.0015 (0.0064)	0.01	probably damaging	Yes (21698661, 22826602)
21	Painful Sensory neuropathy	Definite	SCN10A	c.3445G>A	p.V1149Mp.Pro1149Met	VUS	0.0001	0.0003	0.0039 (0)	0.00008 (0)	0.00	probably damaging	No studies for this variant
22	Small fibre neuropathy	Definite	SCN10A	c.2428G>T	p.Gly810Trp p.G810W	VUS	0.0003	0.0005	0.0039 (0)	0.0004 (0)	0.00	probably damaging	No studies for this variant

23	Painful Sensory neuropathy	Definite	SCN10A	c.2737G>A	p.Ala913Thr p.A913T	VUS	0.0003	0.0006	0.0039 (0)	0.0003 (0)	0.01	probably damaging	No studies for this variant
24	Erythromelalgia	Probable	SCN10A	c.968A>G	p.Y323Cp.Tyr323Cys	VUS	.	0.00004	0.0039 (0)	0 (0)	0.00	probably damaging	No studies for this variant
25	Small fibre neuropathy	Definite	SCN11A	c.2471A>G	p.Glu824Glyp.E824G	VUS	.	0.00004	0.0039 (0)	0 (0)	0.02	possibly damaging	No studies for this variant
26	Episodic pain	Possible	SCN11A	c.1730C>T	p.P577Lp.Pro577Leu	VUS	0.0002	0.0006	0.0039 (0)	0.0005 (0)	0.00	probably damaging	No studies for this variant

Table 3

Table 4

Genomic coordinates and the HGNC gene symbol for gene models	**Fraction with rare variants**	**Number of variants considered**	**P Value**	**Rho**
Europeans				
Tier 1 pain genes				
All Neuropathic pain (n=126) vs Controls (n=5096)				
1:115828713-115836247_NGF	0.0026346	14	2.053e-08	0
3:38387651-39150434_SCN11A_promoter	0.0037214	186	1.7809e-06	0
Tier 2-3 pain genes				
All Neuropathic pain vs Controls				
1:212733740-213037331_FLVCR1_promoter	0.07838	704	1.0404e-05	0
2:241653459-241759532_KIF1A	0.038202	180	8.74e-08	0
2:241757820-241808205_KIF1A_promoter	0.011691	182	1.5858e-06	0
12:52056606-52201160_SCN8A	0.0097151	44	5.0888e-08	0
2:234835229-234916724_TRPM8	0.017948	90	2.9788e-06	0
Tier 1-3 pain genes				
Probable/Definite Neuropathic pain vs Controls				
1:212733740-213037331_FLVCR1_promoter	0.078509	704	2.0274e-07	0
2:241656788-241737150_KIF1A	0.038265	180	2.7235e-09	0
2:241757820-241808205_KIF1A_promoter	0.01171	182	2.4975e-07	0
1:115828713-115836247_NGF	0.002639	14	2.6429e-09	0
12:52056606-52201160_SCN8A	0.0097312	44	1.5018e-09	0
2:167227721-167351474_SCN9A_promoter	0.037605	461	2.3805e-05	0
8:72935185-72987631_TRPA1	0.015009	73	5.2482e-05	0
2:234835229-234916724_TRPM8	0.017978	90	2.825e-07	0
***SCN9A, SCN10A, SCN11A* functionally validated variants**				
3:38739727-38793804_SCN10A (Small fibre neuropathy)	0.0013329	2	0.00016219	0

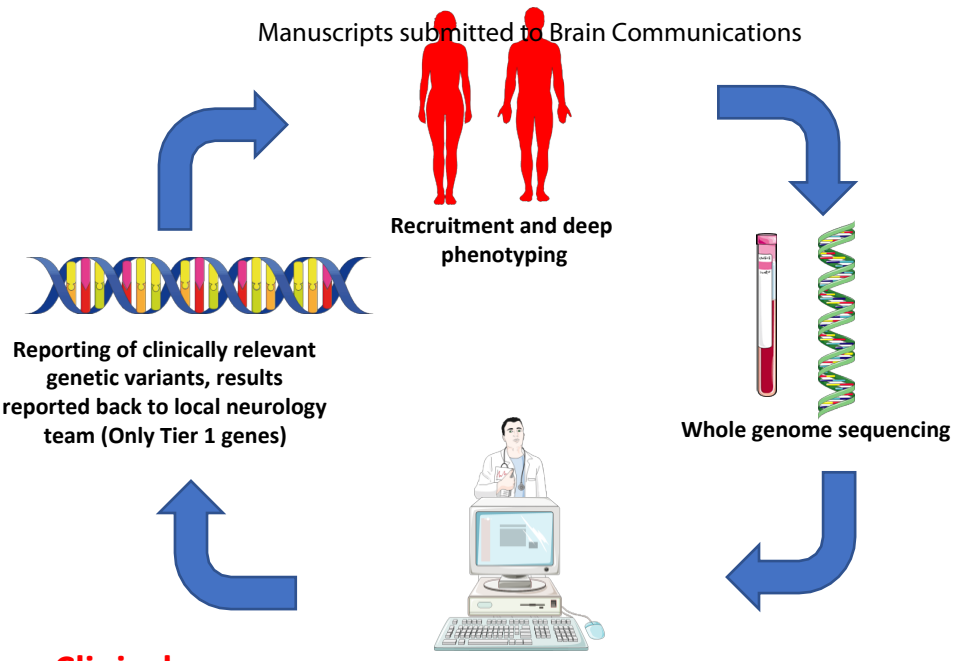
Table 4

Supplementary table 1

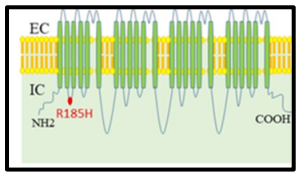
Genomic coordinates and the HGNC gene symbol for gene models	Fraction with rare variants	Variants considered	P Value	Rho
Tier 1-3 pain genes				
Europeans				
Probable/Definite Neuropathic pain vs Controls				
1:212733740-213037331_FLVCR1_promoter	0.078509	704	2.0274e-07	0
2:241656788-241737150_KIF1A	0.038265	180	2.7235e-09	0
2:241757820-241808205_KIF1A_promoter	0.01171	182	2.4975e-07	
1:115828713-115836247_NGF	0.002639	14	2.6429e-09	0
12:52056606-52201160_SCN8A	0.0097312	44	1.5018e-09	0
2:167227721-167351474_SCN9A_promoter	0.037605	461	2.3805e-05	0
8:72935185-72987631_TRPA1	0.015009	73	5.2482e-05	0
2:234835229-234916724_TRPM8	0.017978	90	2.825e-07	0
Possible Neuropathic pain vs Controls				
5:10237637-10311587_CCT5_promoter	0.043829	390	2.1646e-10	0
22:19158112-19468896_CLTCL1_promoter	0.12628	755	1.8541e-05	0
2:27272295-27888854_MPV17_promoter	0.14408	1039	1.1537e-06	0
9:132888940-133713656_PRDM12_promoter	0.12242	778	2.5937e-24	0
3:38887266-38992020_SCN11A	0.022166	116	3.2814e-17	0
3:38387651-39150434_SCN11A_promoter	0.036776	182	9.13e-36	0
2:167051740-167168308_SCN9A	0.031066	138	1.0185e-11	0
9:94179481-95733983_SPTLC1_promoter	0.17162	1253	1.4402e-20	0
14:77972340-78045374_SPTLC2	0.023678	113	7.4671e-17	0
17:16188184-16396312_TRPV2_promoter	0.16927	1239	2.8037e-07	0
14:23990197-24004569_ZFHX2	0.037615	195	1.8008e-11	0
Idiopathic vs Controls				
9:111694228-111884278_ELP1_promoter	0.099514	665	0.00010639	0
1:212733740-213037331_FLVCR1_promoter	0.078238	689	3.5673e-11	0
9:111637167-111693371_IKBKAP	0.021612	98	6.0954e-14	0
2:27532377-27548436_MPV17	0.0065338	31	2.3621e-11	0
2:27272295-27888854_MPV17_promoter	0.14492	1039	1.8079e-11	0
17:40688345-40696166_NAGLU	0.0065338	31	2.5867e-11	0
1:156735601-156831188_NTRK1_promoter	0.053443	606	1.2915e-12	0
3:38387651-39150434_SCN11A_promoter	0.03669	181	7.875e-07	0
12:52056606-52201160_SCN8A	0.0093818	42	7.6272e-10	0
2:167227721-167351474_SCN9A_promoter	0.03736	445	3.7788e-07	0
9:94794766-94877614_SPTLC1	0.0073714	41	2.0265e-12	0
8:72935185-72987631_TRPA1	0.01491	72	2.1457e-10	0
Non-freezing cold injury vs Controls				
11:62605687-64041758_ATL3_promoter	0.2605	1807	1.9334e-26	0
22:19158112-19468896_CLTCL1_promoter	0.12622	755	1.7755e-11	0
6:56322787-56765386_DST	0.064706	326	7.2668e-21	0
2:241653211-241737219_KIF1A	0.037479	174	2.6565e-24	0
2:27272295-27888854_MPV17_promoter	0.14437	1036	2.418e-05	0
1:115828571-115836350_NGF	0.002521	13	6.9657e-126	0
1:115257831-115881445_NGF_promoter	0.073613	415	9.0969e-55	0
12:51791830-52209151_SCN8A_promoter	0.073613	593	5.8951e-06	0
9:94179481-95733983_SPTLC1_promoter	0.17143	1244	5.4476e-30	0
14:77768191-78083941_SPTLC2_promoter	0.06084	683	1.1154e-26	0
2:234825742-235408070_TRPM8_promoter	0.058824	421	9.7579e-49	0
12:109567961-110487716_TRPV4_promoter	0.16185	898	1.6927e-12	0
Erythromelalgia vs Controls				
5:10254263-10266494_CCT5	0.013754	63	1.1515e-09	0
19:9647679-11208553_DNMT1_promoter	0.44918	3552	5.0184e-07	0
6:56322787-56765386_DST	0.064743	328	9.2064e-09	0
9:111694228-111884278_ELP1_promoter	0.099631	660	2.6704e-12	0

1:212733740-213037331_FLVCR1_promoter	0.078665	695	4.5875e-62	0
12:5020374-5027410_KCNA1	0.022979	114	8.8721e-09	0
20:43720954-43728938_KCNS1	0.016437	79	5.6901e-10	0
20:43593848-44003217_KCNS1_promoter	0.046964	245	6.3713e-22	0
2:241653211-241737219_KIF1A	0.037571	174	9.983e-40	0
2:27272295-27888854_MPV17_promoter	0.14425	1040	4.0699e-07	0
1:115257831-115881445_NGF_promoter	0.073465	414	4.0189e-19	0
3:38738320-38835432_SCN10A	0.024992	117	2.745e-08	0
12:52078070-52202289_SCN8A	0.0093928	42	6.9227e-19	0
2:167051740-167168308_SCN9A	0.03103	138	0.00011167	0
17:75303232-75496662_SEPT9	0.020798	92	9.6699e-10	0
17:74347488-75883645_SEPTIN9_promoter	0.68031	14656	2.0928e-23	0
9:94179481-95733983_SPTLC1_promoter	0.17159	1249	1.658e-17	0
2:234835229-234928166_TRPM8	0.017612	88	5.975e-10	0
12:936533-1020561_WNK1	0.030191	130	9.9286e-06	0
Post Traumatic Neuropathy vs controls				
14:50777141-51327966_ATL1_promoter	0.094511	689	3.6407e-14	0
11:62605687-64041758_ATL3_promoter	0.2607	1807	7.2586e-06	0
19:9647679-11208553_DNMT1_promoter	0.44972	3553	2.0827e-11	0
1:46215085-47268057_FAAH_promoter	0.10307	553	5.4233e-06	0
9:111629802-111693471_IKBKAP	0.021655	96	1.8508e-18	0
20:43720954-43728938_KCNS1	0.016451	79	1.9211e-10	0
2:241653211-241737219_KIF1A	0.037771	176	2.0856e-38	0
9:132888940-133713656_PRDM12_promoter	0.12187	777	4.1157e-06	0
3:38387651-39150434_SCN11A_promoter	0.036596	182	7.715e-09	0
12:51791830-52209151_SCN8A_promoter	0.073527	596	4.1392e-20	0
2:234835229-234928166_TRPM8	0.017626	88	1.6971e-24	0
18:29077164-29267441_TTR_promoter	0.077052	399	2.057e-07	0
12:678629-1060004_WNK1_promoter	0.1296	1229	3.4161e-11	0
Neuropathic Pain Not Otherwise Specified vs controls				
5:10237637-10311587_CCT5_promoter	0.043807	390	1.7269e-05	0
6:56322787-56765386_DST	0.064787	328	1.2338e-16	0
1:212733740-213037331_FLVCR1_promoter	0.078214	689	3.2979e-10	0
1:115257831-115881445_NGF_promoter	0.073347	411	5.1011e-05	0
1:156735601-156831188_NTRK1_promoter	0.053206	606	4.9332e-07	0
9:132888940-133713656_PRDM12_promoter	0.12219	777	5.1652e-10	0
3:38387651-39150434_SCN11A_promoter	0.036925	183	1.1968e-45	0
12:52078070-52202289_SCN8A	0.0093991	41	9.7448e-23	0
2:167051740-167168308_SCN9A	0.031051	137	1.1055e-07	0
2:167227721-167351474_SCN9A_promoter	0.037261	441	1.2761e-07	0
9:94179481-95733983_SPTLC1_promoter	0.17153	1253	9.4963e-18	0
14:77972340-78045374_SPTLC2	0.023666	113	4.924e-13	0
Africans				
Post Traumatic Neuropathy vs controls				
6:56323823-56819337_DST	0.076923	14	8.3758e-05	0
Small Fibre Neuropathy vs controls				
2:241653211-241737219_KIF1A	0.044586	7	1.9638e-06	0
2:234835229-234928166_TRPM8	0.044586	7	6.7973e-06	0
12:936533-1020561_WNK1	0.031847	5	0.00010244	0
12:678629-1060004_WNK1_promoter	0.17834	64	6.2972e-06	0
Non-freezing cold injury vs Controls				
1:156811877-156851636_NTRK1	0.02139	4	7.1487e-09	0

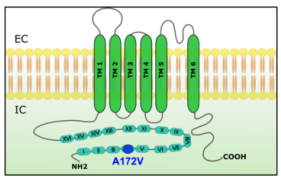
Supplementary table 1



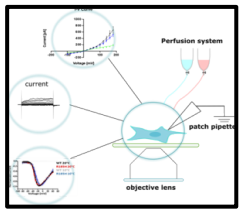
Increase frequency of *SCN9A* p.Arg185His in patients with non-freezing cold injury

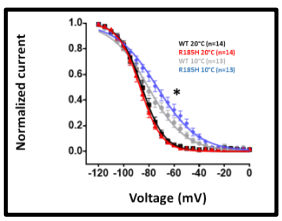


Identification of novel *TRPA 1* variant p.Ala172Val in episodic pain disorder

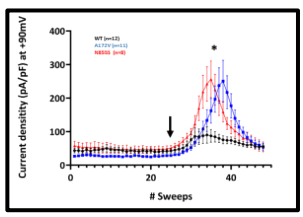


Patch clamp analysis





Gain of function of $Na_V1.7$ after cooling (the environmental trigger for non-freezing cold injury)



Gain of TRPA 1 channel function in response to agonist stimulation.

Abbreviated summary

‘Whole genome sequencing revealed clinically relevant variants in 12% of participants with extreme neuropathic pain disorders. The majority of variants were in ion channels including new phenotype associations (*SCN9A* p.Arg185His and non-freezing cold injury) and novel gain of function variants (*TRPA1* p.Ala172Val and episodic pain).’

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No.	Recommendation	Page No.	Relevant text from manuscript
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract		
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3	
Introduction				
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4	
Objectives	3	State specific objectives, including any prespecified hypotheses	4	
Methods				
Study design	4	Present key elements of study design early in the paper	5-10	
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5	
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5, table 1	
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case		
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5-10	
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5-10	
Bias	9	Describe any efforts to address potential sources of bias	5-10	
Study size	10	Explain how the study size was arrived at	Not applicable	

Continued on next page

Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	5-10	
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6,7,10	
		(*b*) Describe any methods used to examine subgroups and interactions	5-10	
		(*c*) Explain how missing data were addressed	Not applicable	
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	6-8	
		(*e*) Describe any sensitivity analyses		
Results				
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	11	
		(b) Give reasons for non-participation at each stage	Figure 1	
		(c) Consider use of a flow diagram	Figure 1	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	11-14	
		(b) Indicate number of participants with missing data for each variable of interest		
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)		
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time		
		Case-control study—Report numbers in each exposure category, or summary measures of exposure		
		Cross-sectional study—Report numbers of outcome events or summary measures	Table 2, 11-14	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Figure 2-4; table	
		(*b*) Report category boundaries when continuous variables were categorized		
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period		

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Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	11-12	
Discussion				
Key results	18	Summarise key results with reference to study objectives	15	
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	15-18	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	15-18	
Generalisability	21	Discuss the generalisability (external validity) of the study results	15,16,18	
Other information				
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	19,20	

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.