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RESEARCH ARTICLE

Regulation of Naᵥ1.7: A Conserved SCN9A Natural Antisense Transcript Expressed in Dorsal Root Ganglia

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Abstract

The Naᵥ1.7 voltage-gated sodium channel, encoded by SCN9A, is critical for human pain perception yet the transcriptional and post-transcriptional mechanisms that regulate this gene are still incompletely understood. Here, we describe a novel natural antisense transcript (NAT) for SCN9A that is conserved in humans and mice. The NAT has a similar tissue expression pattern to the sense gene and is alternatively spliced within dorsal root ganglia. The human and mouse NATs exist in cis with the sense gene in a tail-to-tail orientation and both share sequences that are complementary to the terminal exon of SCN9A/Scn9a. Overexpression analyses of the human NAT in human embryonic kidney (HEK293A) and human neuroblastoma (SH-SY5Y) cell lines show that it can function to downregulate Nav1.7 mRNA, protein levels and currents. The NAT may play an important role in regulating human pain thresholds and is a potential candidate gene for individuals with chronic pain disorders that map to the SCN9A locus, such as Inherited Primary Erythromelalgia, Paroxysmal Extreme Pain Disorder and Painful Small Fibre Neuropathy, but who do not contain mutations in the sense gene. Our results strongly suggest the SCN9A NAT as a prime candidate for new therapies based upon augmentation of existing antisense RNAs in the treatment of chronic pain conditions in man.

Introduction

Following the cataloguing of the human genome and transcriptome it has become apparent that there are probably more genes in the human genome that encode regulatory RNAs than
those that encode proteins [1]. One major class of regulatory RNA genes contains the long non-coding RNAs (lncRNAs), of which natural antisense transcripts (NATs) are an important subset. NATs can be defined as processed transcripts that are complementary to the corresponding processed sense transcript in exonic regions [2]. NATs can exist in cis or trans to the target gene and are relatively common, with approximately 70% of all genomic loci showing evidence of transcription from both sense and antisense strands [3]. Prominent examples of NATs include Tsix (the NAT for Xist), Wrap53 (the NAT for p53) and BACE1-AS (the NAT for beta-secretase-1) [4–6]. In the pain field, a NAT was recently reported for the voltage-dependent potassium channel Kcna2 [7]. This NAT is expressed in rat dorsal root ganglion (DRG) neurons and is upregulated in response to peripheral nerve injury. The increase in NAT levels downregulates Kcna2, attenuating total voltage-gated potassium currents, increasing excitability in DRG neurons and producing neuropathic pain symptoms.

We were interested to discover whether a NAT exists for SCN9A, another pain-related gene, which encodes the Na1.7 voltage-gated sodium channel. Previously we reported that recessive loss of function mutations in this channel result in a complete inability to perceive pain (CIP) [8]. In addition to being pain-free from birth, SCN9A-CIP patients also lack a sense of smell, but are otherwise normal [9]. Consequently, this channel has been identified as a promising target in the pharmaceutical industry for the development of new analgesic drugs [10]. In contrast to the pain-free phenotype, there are also debilitating painful Mendelian disorders resulting from gain of function of Na1.7, such as Inherited Primary Erythromelalgia (IEM), Paroxysmal Extreme Pain Disorder (PEPD) and painful small fibre neuropathy [11–13]. We considered that if a NAT did exist for SCN9A, then perhaps it played a role in regulating Na1.7 protein levels and hence altering responses to painful stimuli.

In this study, using an in silico approach to inform the design of RT-PCR reactions, we have cloned a NAT for SCN9A that is conserved in humans and mice. The tissue expression profile of the NAT is similar to the sense gene, indicating that it may play an important functional role. Overexpression analyses of the NAT have shown that it reduces Na1.7 mRNA, protein and currents. This NAT is therefore a potentially interesting candidate gene for IEM, PEPD and small fibre neuropathy patients that lack pathogenic mutations in SCN9A [14,15].

Results

Cloning the SCN9A/Scn9a natural antisense transcripts

In silico analyses of the human and mouse SCN9A/Scn9a gene footprints using the UCSC genome browser identified several expressed sequence tags (ESTs) that were partially complementary to exonic regions of the sense gene. Alignment of the longest human EST, BC051759, to the genomic sequence indicated a cDNA comprised of 12 exons; four of which were complementary to and partly or wholly overlapped exons from SCN9A (S1 Fig); and with five exons containing SINE and/or LINE repeat sequences. Exons were flanked with the canonical AG-GT splice acceptor and donor sites and the final exon contained an AAUAAA polyadenylation signal. In Genbank the assembly of ESTs has subsequently been annotated as LOC101929680 (NR_110260), which spans 220 kb on chromosome 2 and encodes an uncharacterized long non-coding RNA of 2305 bp (Fig 1A). Using human dorsal root ganglion cDNA as template we amplified two alternative splice variants, which were submitted to Genbank. Compared to NR_110260, the first splice variant (KM096550) excludes exon 2 and uses an alternative splice acceptor site within exon 7. The second splice variant (KM096551) excludes both exon 2 and exon 8 (Fig 1A). Interestingly, some SCN9A point mutations previously shown to cause the human monogenic pain disorders CIP, IEM and PEPD also change the sequence of the NAT (S1 Fig).
Analysis of the mouse genome also led to the identification of several ESTs that were antisense to *Scn9a*. For example, EST AK138532 indicated a cDNA comprised of four exons, one of which splices into a LINE repeat and two of which overlap *Scn9a* sense gene exons (NR_033495; Fig 1B). Similar to the human *SCN9A* NAT, exons were flanked with canonical AG-GT splice acceptor and donor sites and the final exon contained an AAUAAA polyadenylation signal. Both the human and mouse NATs contain sequences that overlap the final sense *SCN9A*/*Scn9a* exon, potentially indicating a conserved regulatory function of these NATs in man and mouse. Using mouse dorsal root ganglion cDNA as template we amplified the identical sequence to NR_033495 (KM096552, Fig 1B) as well as a splice variant (KM096553, Fig 1B), which uses an alternative splice donor site in exon 3.

*In silico* translation of the human and mouse NAT sequences shows that the longest potential open reading frames are 67 and 114 amino acids respectively (S2 Fig). The lack of a long
open reading frame and the poor codon conservation is consistent with the definition of a long non-coding RNA [1].

Scn9a sense and NAT genes have a similar tissue expression profile

To investigate the tissue expression profile of the Scn9a NAT compared with the sense gene, we ran qPCR assays across a range of mouse tissue cDNA samples (Fig 2). The Scn9a sense and NAT genes have a relatively restricted expression pattern and are co-expressed in adult brain, DRG and spinal cord tissues. In addition, the NAT also shows expression within adult eye. The co-expression of the sense and NATs in similar tissues suggests that the NAT could have a direct regulatory effect on Scn9a gene functions. To further understand the relative expression of the NAT and Scn9a within DRG neurons, we analysed data from a recent paper in which DRG neurons have been categorized into 11 subtypes based on single-cell RNA-seq expression data (S3 Fig) [16]. This shows that the NAT is expressed in six of the eleven DRG neuronal subtypes. Interestingly, in the remaining five DRG subtypes with no NAT expression detected, there is robust Scn9a expression. Conversely, in the only neuronal subtype without Scn9a expression (‘NF5’), there is relatively high expression of the NAT, indicating that within particular neuronal cell populations there are contrasting expression profiles of the NAT and Scn9a.
Overexpression of the human NAT specifically reduces Nav1.7 peak sodium currents

To functionally test the effects of overexpressing the human NAT on sodium currents, HEK293 cells stably expressing either Nav1.7 or Nav1.6 were transfected with (1) NAT in pcDNA3 plus a GFP-expressing vector (pEGFP-N1) or (2) empty pcDNA3 plus pEGFP-N1. Two days after transfection the green fluorescing cells were patch clamped. Overexpression of the human SCN9A NAT in the Nav1.7 stable cell line resulted in a statistically significant reduction in the peak sodium current (Fig 3). In Nav1.6 stably expressing cells, overexpression of the NAT had no effect on the sodium current, indicating that the NAT specifically affects the activity of Nav1.7.

Stable expression of the human NAT in SH-SY5Y neuroblastoma cells

The SH-SY5Y neuroblastoma cell line endogenously expresses the SCN9A gene [17] but does not co-express the NAT (Fig 4A). We generated a SH-SY5Y cell line that stably expresses the human NAT cDNA under the control of a CMV promoter to assess the effect of overexpression of the NAT on endogenously-expressed Na+,1.7. The SCN9A mRNA level in the NAT-stable cell line was significantly downregulated compared to wild-type SH-SY5Y cells (Fig 4B). Furthermore, patch clamping of this cell line showed a statistically significant reduction in the peak sodium current compared to a SH-SY5Y cell line that did not express the human NAT (Fig 4C and 4D). Voltage-current relationships were unaltered between the two cell lines.
In addition to sharing overlapping sequences with SCN9A, the NAT also contains sequences overlapping with two exons from each of SCN1A, SCN2A and SCN3A (S4 Fig). As the NAT shows high expression in the brain it is possible that the NAT may also regulate the
Na\(_{1.1}\)-Na\(_{1.3}\) brain-expressed sodium channels encoded by \textit{SCN1A}-\textit{SCN3A}. We were therefore interested to determine whether overexpression of the NAT downregulated endogenously expressed Na\(_{1.1}\), Na\(_{1.2}\) or Na\(_{1.3}\) in the NAT-stable SH-SY5Y cell line. Real-time qPCR showed the expression level of \textit{SCN2A} and \textit{SCN3A} were not significantly different between the naïve SH-SY5Y cells (sham) and the stable-NAT SH-SY5Y cells (\textit{SCN1A} expression could not be detected in the SH-SY5Y cell lines) (S5 Fig), further indicating that the functional effects of the NAT are \textit{SCN9A}-specific.

Next, the effect of NAT overexpression on Na\(_{1.7}\) protein levels was investigated. A HEK293 cell line was generated which stably expressed an epitope-tagged (TAP) Na\(_{1.7}\) under the control of a CMV promoter. This cell line was transiently transfected with the human NAT, and anti-FLAG coupled Dynabeads were used to pull down TAP-tagged Na\(_{1.7}\), which was subsequently detected on a western blot using an anti-HAT antibody. Comparison of NAT transfected and sham transfected cells showed that the transient NAT transfection resulted in a reduction in TAP-tagged Na\(_{1.7}\) protein levels (Fig 4E). This reduction in protein level was further confirmed by immunoblotting using a pan sodium channel antibody (S6 Fig).

\textbf{NAT and \textit{Scn9a} mRNA levels are unchanged in inflammatory and neuropathic pain states}

Given that the NAT specifically downregulates human Na\(_{1.7}\) protein levels and attenuates its currents, we investigated whether the NAT has a role \textit{in vivo} to regulate mouse \textit{Scn9a} mRNA levels in different pain states. Two different inflammatory pain mouse models (carrageenan and complete Freund’s adjuvant (CFA)) and one neuropathic pain model (chronic constriction injury) were used. Injection of carrageenan into the hind paw induces an acute and highly reproducible inflammatory response resulting in oedema, hyperalgesia and erythema that can persist for 6 days [18]. Likewise, injection of CFA results in acute thermal and mechanical hyperalgesia that can persist for more than 2 weeks. In the chronic constriction injury model, sutures are tied around the sciatic nerve which leads to thermal and mechanical hyperalgesia presenting within the first week and persisting for several weeks. Real-time qPCR assays using RNA isolated from ipsilateral L4-L6 dorsal root ganglia dissected 3 days (CFA), 2 hrs and 24 hrs (carrageenan) and 2 weeks (CCI) following the start of each pain model showed that neither \textit{Scn9a} sense nor NAT mRNA levels change significantly (Fig 5A–5C).

\textbf{Discussion}

We have identified, cloned and characterized a human and mouse natural antisense transcript for the \textit{SCN9A} gene. Both human and mouse NATs are organised in a tail-to-tail configuration with the sense gene and both show evidence of alternative splicing, which is consistent with other reported NATs [1]. Although the mouse \textit{Scn9a} NAT has fewer exons than the human NAT, both contain conserved sequences that overlap the final sense \textit{SCN9A}/\textit{Scn9a} exon. Given this conserved gene structure and the similar tissue expression profile of the sense and NAT genes, we hypothesized that the NAT regulates the function of \textit{SCN9A}. We overexpressed the NAT in human cell lines that endogenously expressed Na\(_{1.7}\) and in cells that stably expressed from a CMV promoter either Na\(_{1.7}\) or epitope-tagged Na\(_{1.7}\). Real-time qPCR, immunoblotting and electrophysiological assays showed a significant reduction in Na\(_{1.7}\) mRNA, protein levels and currents.

How do NATs regulate the function of their sense gene counterparts? Characterization of other NATs has ascribed to them diverse cellular functions including transcriptional collision, RNA interference, regulation of alternative splicing, RNA editing, epigenetic regulation, RNA masking, translation inhibition and mRNA destabilisation [19]. In our experimental set-up we
overexpressed the processed NAT from a CMV promoter in cell lines. This led to a reduction in sense mRNA and consequently a reduction in functional Nav1.7 protein. The NAT might function by downregulating SCN9A transcription, for example by guiding chromatin-modifying enzymes to the SCN9A promoter region. Alternatively, overexpression of the NAT could
be leading to the generation of endogenous short RNAs (endo-siRNAs) thus leading to promotion of the RNA interference pathway [20]. A third mechanism could be that the NAT:sense mRNA duplexes are promoting destabilisation and degradation of the sense mRNA and/or inhibiting translation. A greater insight into the function of the NAT in vivo would be gleaned from the creation of a NAT knockout mouse. In a comparable experiment, Komine et al. generated a Zfhx2-AS knockout mouse through deletion of the transcriptional start site region for its corresponding NAT [21]. Normally, the Zfhx2 expression pattern in the developing brain is complementary to the expression of its NAT. However in the Zfhx2-AS knockout mouse, the expression of the Zfhx2 homeobox-containing transcription factor becomes dysregulated. It would be interesting to determine whether the expression pattern of Scn9a is altered in a Scn9a NAT knockout mouse. Furthermore, the effects on transcription of other genes, which may also be potentially regulated by the NAT, could also be investigated in a NAT knockout, for example Scn1a and Scn7a, which reside in the same chromosomal region as Scn9a.

Detailed analyses of Scn9a knockout mice have shown that acute, inflammatory and some forms of neuropathic pain require the expression of Na\textsubscript{v}1.7 [22–24]. We hypothesized that the NAT may be downregulated in pain states, hence leading to upregulation of SCN9A. To test this, we assessed mRNA levels of the sense and NAT genes in CFA and carrageenan-induced models of inflammatory pain and in the chronic constriction injury neuropathic pain model. This showed that the mRNA level of neither the sense nor the NAT gene was significantly altered. The lack of change in the sense gene mRNA was a surprising finding given that, for example, Na\textsubscript{v}1.7 protein levels have previously been shown to increase following carrageenan injections [25]. Further work on epitope-tagged Na\textsubscript{v}1.7 knockin mice using anti-FLAG/HAT antibodies should help to confirm whether protein levels change in different pain models. Furthermore, intrathecal NAT overexpression by viral delivery could help to determine whether the NAT is able to confer analgesia and reduce the pain experienced in different inflammatory and neuropathic pain states.

In summary, this DRG-expressed natural antisense transcript attenuates native sodium currents, is co-expressed with its corresponding gene, and consequently has the potential to regulate pain thresholds via transcriptional and post-transcriptional regulation of SCN9A. Given that the presence of the NAT reduces Na\textsubscript{v}1.7 currents, then intuitively the lack of the NAT may increase sodium currents. This could lead to increased excitability of damage-sensing neurons, enabling a fine-control of responses to painful stimuli. Other regulatory mechanisms mediated by the NAT are also plausible, as discussed above. It will be interesting to determine whether loss of function mutations in the NAT are responsible for inherited painful disorders that map to human chromosome 2, and whether SNPs within the NAT alter pain thresholds in the general population.

Material and Methods

Cloning the human and mouse NATs

Human dorsal root ganglia total RNA was purchased from Clontech; mouse total RNA was isolated from DRGs dissected from wild-type C57BL/6 mice using the RNeasy MinElute Kit (QIAGEN). Total RNA was reverse transcribed into cDNA using Superscript III (Life Technologies) and oligo d(T), according to the manufacturer’s instructions. The human SCN9A NAT was PCR-amplified from DRG cDNA using KAPA HiFi DNA polymerase (Kapa Biosystems) or LA Taq (TaKaRa) in three overlapping fragments using the following primers: Fragment 1, 5’ GAA TGA AAT TTA GTG TTT CCC ATC C, 5’ AGC AAT GTT TCA CCT CCA GAG ATC; Fragment 2, 5’ GGA ATT CAG GCA AAG TTG GA, 5’ CAC CAA CAT TCA GCC ATT TG; Fragment 3, 5’ CTG ATT ATT GGG AGA CTT TTG GAG, 5’ GCT CCT ATT TCT GAG.
TTT ATA CTG TG. The mouse Scn9a NAT was PCR-amplified using the primers 5’ agc aag agt aag aag tat tgg c, 5’ cat tat att tca ttt taa tg. PCR products were cloned using the Zero Blunt TOPO PCR Cloning Kit (Life Technologies). Individual colonies were Sanger sequenced and submitted to GenBank under the accession numbers KM096550 (clone LA5), KM096551 (clone LA8), KM096552 (clone 3.2) and KM096553 (clone 3.1).

Real-time qPCR assay for mouse tissue panel

One μg of total RNA derived from a range of tissues (Clontech) or prepared in-house from DRG was reverse-transcribed into cDNA using Superscript III (Life Technologies) and oligo d (T). Real-time qPCR using technical triplicates was performed using SYBR Green (Applied Biosystems) and the following primers: Mouse Scn9a 5’ GAG GGG CAA ACT GAC TAC A and 5’ AGA AAC ATT CCT ACA ATG GAG (efficiency 1.91); Mouse NAT 5’ TGC TGT CAA CTC CTG AAC CA and 5’ TCC AAC TTT GCC ACA ATG AG (efficiency 1.96); Mouse Actb 5’ TTC TTT GCA GCT CCT TCG TT and 5’ ATG GAG GGG AAT ACA GCC C (efficiency 1.83). Relative expression of the target gene was calculated using the comparative ΔΔCT method [26,27]. Briefly, expression of the test gene was compared with that of Actb measured on the same sample, giving a CT difference (ΔCT) for Actb minus the test gene. The relative expression of Scn9a and the NAT in specific tissues was calculated in relation to the expression levels in DRG using the comparative ΔΔCT method [26]. ΔCT values of individual tissues were compared to ΔCT of DRG, giving ΔΔCT values of a specific tissue and relative expression was calculated as 2\(^{-ΔΔCT}\). Mean, standard error and statistics were calculated from the ΔΔCT data. Data were analysed using Microsoft Excel, and statistics were calculated using GraphPad Prism 5.01 software (GraphPad Software, San Diego, CA).

Cloning the human NAT into an expression vector

The human SCN9A NAT was PCR-amplified from IMAGE clone 5582960 (BC051759) using Phusion High-Fidelity DNA polymerase (NEB) and primers 5’ CCC AAG CTT gtc tta gtc ctc tga ata ttt t and 5’ CGG GGT ACC CCA ATT GAT GGA GAA TTT TAT. The PCR product was then cloned into the HindIII and KpnI restriction sites of pcDNA3 (Life Technologies) and fully sequenced. The cloning was designed so that the NAT was inserted at the most 5’ end of the multiple cloning site and near to the transcription start site of the vector, to minimise the additional nucleotides that were added to the 5’ end of the transcribed long ncRNA.

Transfection of cell lines

HEK293 cells stably expressing human Na\(_{\alpha}1.7\) plus SCN1B (L11) or Na\(_{\alpha}1.6\) plus SCN1B (L40) (Scottish Biomedical) were grown according to the manufacturer’s instructions and passaged four times before transfections began. Cells were split into 3.5 cm dishes and grown to 95% confluency. On the day of transfection, cells were transfected with 250 ng of pEGFP-N1 (Clontech) plus 1000 ng of human NAT in pcDNA3 or empty pcDNA3 using a ratio of 1 μg DNA:2.5 μl Lipofectamine 2000 (Life Technologies). After 6 hours, the transfected cells were re-seeded at a low density and incubated for 48 hours prior to patch clamp analysis. Two independent transfections were tested.

NAT-SH-SY5Y stable cell line

The human neuroblastoma cell line, SH-SY5Y (Public Health England) was cultured at 37°C/ 5% CO\(_2\) in Ham’s F12:EMEM (1:1) supplemented with 2 mM glutamine, 1% non essential amino acids and 10% foetal bovine serum (Life Technologies). The human NAT in pcDNA3
was linearised with *Pvu* I and following gel purification (Qiagen), 10 ug was transfected into a 10 cm 80% confluent dish of SH-SY5Y cells using Lipofectamine 2000 (Life Technologies). Six hours later, the medium was replaced with the selection antibiotic G418 (500 μg/ml). Cells were monitored on a daily basis until twenty-four discrete colonies could be selected for expansion and screening by RT-PCR. Real-time qPCR was performed using SYBR Green (Applied Biosystems) and the following primers: Human SCN9A 5’ AGA GGG GTA CAC CTG TGT GAA and 5’ CCC AGG AAA ATC ACT AGC ACA AA (efficiency 1.9); Human NAT 5’ GGA GTC ACT GGG ATT AAA GGC AT and 5’ TTC TTT GTC GCT GGT GGC TAG AG (efficiency 2); Human ACTB 5’ CGG CGC CAG CTC ACC ATG and 5’ CAC GAT GGA GGG GAA GAC GG (efficiency 1.85). The expression levels of SCN1A, SCN2A and SCN3A in the SH-SY5Y cells was determined using Taqman assays (Life Technologies) according to the manufacturer’s instructions. The following probes were used SCN1A (Hs00374696_m1), SCN2A (Hs01109877_m1), SCN3A (Hs00366902_m1) and GAPDH (Hs02758991_g1). Relative expression levels of mRNA were calculated using the comparative ΔΔCt (Ct) method and statistical significance was determined using an unpaired t-test.

**Patch clamp analysis of Na\textsubscript{v}1.7-HEK293, Na\textsubscript{v}1.6-HEK293 and NAT-SH-SY5Y stable cell lines**

Whole cell patch clamp recordings were made from Na\textsubscript{v}1.7-HEK293, Na\textsubscript{v}1.6-HEK293 and NAT-SH-SY5Y stable cell lines at room temperature. In the case of the transient Na\textsubscript{v}1.7 NAT transfections, whole-cell membrane current recordings were performed 46 to 78 hrs after transfection. Micropipettes were pulled from borosilicate glass capillaries (GC150F-10; Harvard Apparatus, Kent, UK) using a Brown-Flaming P-97 horizontal micropipette puller (Sutter Instruments, Novato, CA, USA) and then fire polished on a microforge (MF-830 Narishige Group, Tokyo, Japan). Voltage errors were minimised with correction and prediction mode of series resistance compensation both set to 50%. Extracellular solution contained (in mmol/L): 140 NaCl, 4 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES, adjusted to pH 7.4 with NaOH, osmolarity 320–325 mOsm/L with glucose. Pipettes were filled with an intracellular solution containing (in mmol/L): 140 CsCl, 5 NaCl, 5 EGTA, 2 MgCl\textsubscript{2}, 10 HEPES adjusted to pH 7.3 with CsOH, osmolarity 305–310 mOsm/L with glucose. Once filled with the appropriate intracellular solution, recording electrodes had a resistance between 2.0 and 3.0 MΩ. A silver chloride coated silver wire served as a reference electrode with one end connected to the ground input of the amplifier and the tip placed directly into the bath solution. Cells having a leak current after establishing whole-cell configuration of more than 10% of the peak sodium current were discarded and those which had developed leak of this magnitude during the experiment were not used in the final analysis. The liquid junction potential between the bath and the pipette solutions was not corrected. Whole-cell membrane currents were filtered at 5 kHz and sampled at 20 kHz using either an Axopatch 200B patch clamp amplifier or Axon Multiclamp 700B (Molecular Devices, Foster City, CA) and Digidata 1200B A/D converter (Molecular Devices, Foster City, CA). Data were acquired on a Windows-based PC using Clampex (Molecular Devices, Foster City, CA) software and analysed by pCLAMP (Clampfit) 9.2 software (Molecular Devices, Foster City, CA).

To characterize the voltage dependency of steady-state channel activation, currents were evoked by voltage increments of 5 mV from -80 to +40 mV for 10 ms from a holding potential of -120 mV with 5 s between pulses. Peak whole cell currents (pA) were measured in response to a 10 ms voltage step from a holding potential of -120 mV to 0 mV and normalized to cell capacitance (pF).
Na$_v$1.7-TAP tag stable HEK293A cell line

The human SCN9A mammalian expression construct, FLB [8] was modified by cloning in a sequence encoding a TAP tag (peptide: SRK DHL IHN VHK EEH AHA HNK IEN LYF QGE LPT AAD YKD HDG DYK DHD IDY KDD DDK) immediately prior to the stop codon. The TAP tag at the extreme C-terminus of Na$_v$1.7 is comprised of a HAT domain and 3 FLAG tags, enabling immunodetection with either anti-HAT or anti-FLAG antibodies. The Na$_v$1.7-TAP stable HEK293A cell line was generated using the same methods as described for the NAT-SH-SY5Y stable cell line. For the immunoprecipitation experiments, the Na$_v$1.7-TAP stable HEK293A cell line was firstly transiently transfected with the human NAT expression construct or an empty pcDNA3 control (as described above) and cells collected 48 hrs later. Protein was isolated using RIPA buffer and equal amounts loaded onto 100 ul anti-FLAG (Sigma, F1804) coupled Dynabeads (Life Technologies), according to the manufacturer’s instructions. Samples were then boiled in Laemmli buffer and equal volumes loaded onto 4–12% polyacrylamide gels. Immunopurified Na$_v$1.7-TAP was detected on immunoblots using an anti-HAT antibody (LSBio, LS-C51508). In addition, equal amounts of crude lysate protein were subjected to immunoblotting and detected using anti-FLAG, anti-alpha tubulin (Abcam, ab7291), anti-pan sodium channel (SIGMA, S8809) and anti-beta actin (Santa Cruz) antibodies. Densitometry readings were performed using ImageJ software whereby the region of interest (ROI) reading for alpha-tubulin was compared to the ROI reading for the HAT or FLAG bands and subsequently normalized to the sham control.

Murine pain behaviour models

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 with prior approval under a Home Office project license (PPL 70/7382). Two different inflammatory pain models were used: intraplantar injection into the left hind paw of carrageenan or complete Freund’s adjuvant (or 0.9% sodium chloride solution for the sham controls), as described in [18]. The chronic constriction injury (CCI) model of neuropathic pain was also used, in which sutures were tied around the sciatic nerve [18]. All experiments were performed using 6–8 week old male C57BL/6 mice. Total RNA was isolated from dissected L4-L6 ipsilateral DRGs using the RNeasy MinElute Kit (Qiagen). Real-time qPCR was performed using SYBR Green (Applied Biosystems) and the following primers: Mouse Scn9a 5’ GAG GGG CAA ACT GAC TAC A and 5’ AGA AAC ATT CCT ACA ATG GAG (efficiency 1.91); Mouse NAT 5’ TGC TGT CAA CTC CTG AAC CA and 5’ TCC AAC TTT GCC ACA ATG AG (efficiency 1.96); Mouse Gapdh 5’ TGC GAC TTC AAC AGC AAC TC and 5’ CTT GCT CAG TGT CCT TGC TG (efficiency 1.76); Mouse Actb 5’ TTC TTT GCA GCT CCT TGG TT and 5’ ATG GAG GGG AAT ACA GCC C (efficiency 1.83). Relative expression levels of mRNA were calculated using the comparative ΔΔCt (Ct) method and statistical significance was determined using an unpaired t-test.

Supporting Information

S1 Fig. Alignment of SCN9A coding exons (NM_002977) with complementary SCN9A NAT sequence (NR_110260). Na$_v$1.7 protein sequence (NP_002968) and location of human mutations associated with pain disorders highlighted.

S2 Fig. Translation in three frames of the 2 human and 2 mouse NAT splice variants cloned from dorsal root ganglion cDNA. Potential open reading frames beginning with a methionine
residue are highlighted in red. Stop codons are denoted by a hyphen.

S3 Fig. Expression profile (fraction of positive cells by thresholding method) for Scn9a and the NAT (Gm13629) across 11 genetically defined DRG neuronal subtypes. The population size and the fraction of the population that would correspond to one cell are shown at the top. Data taken from Usoskin et al., 2015.

S4 Fig. Alignment of SCN1A exons (NM_001165963), SCN2A exons (NM_021007) and SCN3A exons (NM_006922) with complementary SCN9A NAT sequence (NR_110260). For SCN4A (NM_000334), SCN5A (NM_198056), SCN7A (NM_002976), SCN8A (NM_014191), SCN10A (NM_006514) and SCN11A (NM_014139) there was no significant similarity found with the SCN9A NAT (NR_110260).

S5 Fig. Real-time qPCR showing that the endogenous SCN2A (A) and SCN3A (B) mRNA levels are not significantly different in the stable-NAT SH-SY5Y cell line compared to in naïve SH-SY5Y cells (sham) (n = 3). SCN1A is not expressed in these cell lines.

S6 Fig. Reduction in Na1.7-TAP tag protein levels due to overexpression of the NAT for 48 hrs in a Na1.7-TAP stable HEK293 cell line. Upper: Immunoblot of crude lysate using an anti-pan sodium channel antibody confirms a reduction in Na1.7 protein levels following NAT transfection. Lower: Immunoblot of crude lysate using an antibody to the beta actin housekeeping protein, confirming equal loading.

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Author Contributions

Conceived and designed the experiments: JK RW JEL AMH JV SL NE JZ ALO CGW JNW JJC. Performed the experiments: JK RW JEL AMH JV NE JJC. Analyzed the data: JK RW JEL AMH JV SL NE JNW JJC. Contributed reagents/materials/analysis tools: JK RW JEL AMH SL CGW JNW JJC. Wrote the paper: JK RW JEL AMH JV SL NE JZ ALO CGW JNW JJC.

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