Abstract

Human genetic studies show that the voltage gated sodium channel 1.7 (Na\textsubscript{v}1.7) is a key molecular determinant of pain sensation. However, defining the Na\textsubscript{v}1.7 contribution to nociceptive signalling has been hampered by a lack of selective inhibitors. Here we report two potent and selective arylsulfonamide Na\textsubscript{v}1.7 inhibitors; PF-05198007 and PF-05089771, which we have used to directly interrogate Na\textsubscript{v}1.7’s role in nociceptor physiology. We report that Na\textsubscript{v}1.7 is the predominant functional TTX-sensitive Na\textsubscript{v} in mouse and human nociceptors and contributes to the initiation and the upstroke phase of the nociceptor action potential. Moreover, we confirm a role for Na\textsubscript{v}1.7 in influencing synaptic transmission in the dorsal horn of the spinal cord as well as peripheral neuropeptide release in the skin. These findings demonstrate multiple contributions of Na\textsubscript{v}1.7 to nociceptor signalling and shed new light on...
the relative functional contribution of this channel to peripheral and central noxious signal transmission.

Introduction

Numerous genetic studies implicate Na\textsubscript{v}1.7 in the pathogenesis of distinct pain states (for reviews see [1] [2]). In particular, loss-of-function mutations in SCN9A (the gene encoding Na\textsubscript{v}1.7) have been identified in patients with congenital insensitivity to pain (CIP; [3]), whereas gain-of-function mutations in SCN9A lead to chronic pain syndromes such as paroxysmal extreme pain disorder (PEPD, [4]) and inherited erythromelalgia (IEM) [5] [6] [7] [8]. Moreover, expression of Na\textsubscript{v}1.7 in DRG neurons extends from the peripheral terminals in the skin to the central terminals in the dorsal horn [9]. These studies present a clear link between Na\textsubscript{v}1.7 function and pain sensation and raise the possibility that selective Na\textsubscript{v}1.7 inhibitors might hold therapeutic potential as novel analgesics.

Despite the strong evidence implicating Na\textsubscript{v}1.7 in human pain genetic studies, a detailed investigation of the role of Na\textsubscript{v}1.7 in nociception remains an important area of investigation. Na\textsubscript{v} channels are essential for action potential initiation and upstroke in excitable cells. Out of a repertoire of nine Na\textsubscript{v}s (Na\textsubscript{v}1.1–1.9), five are expressed in varying levels in adult rodent somatosensory DRG neurons: Na\textsubscript{v}1.1, Na\textsubscript{v}1.6, Na\textsubscript{v}1.7, Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9 [10] [11] [12] [13].

Given the participation of multiple Na\textsubscript{v}s in pain signalling, progress in delineating the individual roles of specific Na\textsubscript{v} isoforms in DRG neurons would be accelerated if subtype-selective inhibitors were available.

In this study we characterize two novel arylsulfonamides: a clinical compound, PF-05089771 (for a list of relevant clinical trials see [14]) and a structurally related preclinical tool compound, PF-05198007. Both demonstrate high potency and a high degree of Na\textsubscript{v} subtype selectivity, properties which are gained through a drug/channel interaction that is distinct from that of the classical non-selective pore-blocking drugs such as local anaesthetics. We examined the effects of selective Na\textsubscript{v}1.7 block in both in vitro and in vivo preparations with the principal aim of exploring how Na\textsubscript{v}1.7 influences nociceptor function. Our findings establish a mechanistic basis for Na\textsubscript{v}1.7 contribution to action potential electogenesis in small diameter DRG neurons and describe a functional role for Na\textsubscript{v}1.7 in controlling both neuropeptide release in the peripheral compartment and synaptic transmission in the dorsal horn of the spinal cord.

Materials & Methods

Cell culture

Human embryonic kidney (HEK) 293 cells stably expressing human and mouse Na\textsubscript{v} subtypes were commercially obtained (Millipore). Cells were maintained using minimum essential medium (MEM) with Earle’s salts supplemented by 10% foetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1x non-essential amino acids and 0.4 mg/ml geneticin (G-418) and kept at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}. For manual patch clamp experiments, cells were plated onto glass coverslips and used within 48 hours.

Ethical Approval

Mice were killed by cervical dislocation in accordance with Schedule 1 of the UK Government Animals (Scientific Procedures) Act, 1986, following approval by the Animal Welfare and...
Ethical Review Body, or euthanized by isoflurane in accordance with the National Institutes of Health guide for the Care and Use of Animals following approval by the University of Virginia Institute of Animal Care and Use Committee.

**Mouse DRG preparation**

Dorsal root ganglia (DRG) were isolated and dissociated according to a previously published method [15]. Briefly, DRGs were obtained from all spinal locations and dissociated neurons plated on glass coverslips pre-coated with poly-D-lysine/laminin (BD Biosciences) and left to adhere for 1.5–2 hrs before flooding. Growth media consisted of Lebovitz L-15 Glutamax (Life Technologies) supplemented with 10% FCS, 24 mM NaHCO₃ and 38 mM glucose.

**Human DRG preparation**

Human DRGs (hDRGs) were surgically resected from US organ donors with full legal consent. The hDRG culturing process has been previously described in detail [16]. Briefly, DRG neurons were enzymatically dissociated and maintained in culture for up to 9 days prior to recording. All hDRG tissue culture and experiments on hDRG neurons were conducted at Anabios Corporation (San Diego, CA, USA).

**Molecular Biology/ RNASeq**

Approximately 300 small diameter neurons per sample (n = 4 animals, 2 samples per animal) were picked off coverslips in PBS-/- (phosphate buffered saline without calcium or magnesium) (Invitrogen) using a patch pipette. RNA was extracted using the Qiagen RNeasy micro kit as per the manufacturer’s protocol, including on-column DNase digestion. These control samples were then converted to cDNA and amplified for 18 cycles using the SMARTer v3 kit (Clontech). Libraries were quantified using the Nextera XT kit (Illumina), following a modified protocol (see Fluidigm C1 Single-Cell Auto Prep System manual). This protocol uses lower input amounts than the standard Illumina method; 1.25 μl cDNA at 0.1 ng/μl per fragmentation reaction (5 μl total reaction volume). Negative controls were also prepared from each animal by collecting bath solution; these produced no product at the end of amplification or library preparation and hence were not sequenced. Libraries were quantitated individually using the Qubit High Sensitivity DNA assay (Thermo Fisher Scientific) and library quantification kits (KAPA Biosystems) and pooled in equal amounts for single-end sequencing on an Illumina Nextseq 500. Reads were aligned using STAR (spliced transcripts alignment and reconstruction tool) and assigned to genomic features using Counts and Ensembl gene annotations. Gene read counts were converted to fragments per kilobase per million (FPKM).

**Electrophysiology**

Electrophysiological recordings were obtained from stably transfected HEK 293 cells, acutely dissociated mouse DRG neurons, cultured human DRG neurons or mouse superficial dorsal horn neurons using either an Axopatch 200B or Axopatch 700B amplifier (Molecular Devices), or EPC-10 USB amplifier (HEKA). Small diameter mouse DRG neurons were identified as those with a whole cell capacitance between 10–25 pF [17]. For human DRG neurons, patch clamp recordings were obtained from small-to-medium diameter neurons (<60 μm), the majority of which have previously been classified as nociceptors [16].

Patch pipettes were pulled from thick-walled borosilicate glass (Science Products) and had open tip resistances of 1.5–5 MΩ when filled with intracellular solution (ICS; see below for
composition). Data acquisition was performed using either pClamp v10 (Molecular Devices) or PatchMaster (HEKA) software.

**Voltage clamp.** HEK cells or mouse DRG neurons were continuously superfused with extracellular solution (ECS) containing (in mM): 30 NaCl, 110 Choline Cl, 3 KCl, 0.8 MgCl₂, 1.8 CaCl₂, 0.05 CdCl₂, 10 Glucose, 10 HEPES, 5 Sucrose (300–310 mOsm, titrated to pH 7.4 with TEA-OH). The patch pipette (intracellular) solution (ICS) contained (in mM): 5 NaCl, 135 CsF, 10 CsCl, 2 MgATP, 10 HEPES, 5 EGTA (290–300 mOsm, titrated to pH 7.2 with KOH). For human DRG recordings the following solutions were used (ECS in mM): 150 NaCl, 4 BaCl₂, 2 CaCl₂, 1 MgCl₂, 0.1 CdCl₂, 10 Glucose, 10 HEPES, (300–310 mOsm titrated to pH 7.3 with NaOH). ICS in mM: 140 CsF, 10 NaCl, 1 EGTA, 1 MgCl₂, 10 HEPES, 10 glucose, (290–300 mOsm, titrated to pH 7.3 with Cs-OH). Series resistance compensation was routinely applied to at least 75%. Before acquisition, 20 ms pulses to 0 mV were repeatedly applied (0.05 Hz) from Vm = -120 mV until stable current responses were obtained. All experiments were carried out at room temperature (21–24°C). IC₅₀ values were generated in HEK 293 cell lines by voltage clamping at -120 mV before stepping to the V₀.5 of inactivation for 5 seconds in order to accumulate compound binding. This was followed by a 100 ms return to -120 mV preceding a 20 ms test step to 0 mV. Cells with large TTX-S currents (>5 nA mouse, >8 nA human) and cells with series resistance values greater than 15 MΩ, or variable series resistance were omitted from analysis.

**Current clamp.** ECS contained (in mM) 135 NaCl, 4.7 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES and 10 glucose (300–310 mOsm, pH 7.4 with NaOH). ICS contained (in mM) 140 KCl, 0.5 EGTA, 5 HEPES, and 3 Mg-ATP, (pH 7.3 with KOH, 300–305 mOsm). Single action potentials were evoked from V_m = -70 mV by a 20 ms suprathreshold current step (0.1 Hz).

**Action potential voltage clamp.** Recordings were performed as described in Blair & Bean (2002). Briefly, the internal solution contained (in mM): 140 K-Mes, 13.5 NaCl, 1.6 MgCl₂, 0.09 EGTA, 9 HEPES, 0.9 glucose, 14 Tris-creatine PO₄, 4 Mg-ATP, and 0.3 Tris-GTP (285–300 mOsm, pH 7.2 with KOH). The ECS was identical to that used for current clamp recordings (see above). Action potentials were recorded in current clamp mode following a 20 ms current injection from a V_m of -70 mV. The same cell was then examined under voltage-clamp (-70 mV) whereby the recorded action potential was used as the voltage command. Series resistance was compensated for by up to 80%.

**PatchXpress automated electrophysiological recordings**
Extracellular solution contained (in mM): 40–138 NaCl, 0–98 choline chloride, 2 CaCl₂, 5.4 KCl, 1 MgCl₂, 10 glucose, and 10 HEPES, (295–310 mOsm, pH 7.4 with NaOH). Internal solution contained (in mM): 135 CsF, 5 NaCl, 2 MgCl₂, 10 EGTA, 10 HEPES (285–295 mOsm, pH 7.3 with NaOH). HEK 293 cells constitutively expressing human sodium channels were grown as above to 50–80% confluency and harvested by trypsinization. Trypsinized cells were washed and resuspended in extracellular buffer at a concentration of 10⁶ cells/ml. The onboard liquid handling facility of the PatchXpress was used for dispensing cells and application of test compounds, where washout periods were limited to a maximal duration of 5–10 min. The effect of PF-05089771 was evaluated with voltage protocols identical to those used for conventional patch clamp. A detailed rationale and description of the development of the PatchXpress protocol has been previously described [18]. Previous characterization of the PatchXpress platform demonstrated the ability to generate Naᵥ channel pharmacology comparable to conventional patch clamp data[18].

**Spinal cord slice preparation and electrophysiology**
Male adult CD1 mice were terminally euthanized and the spinal column was removed and submerged in chilled (4°C) artificial cerebrospinal fluid solution (aCSF) comprised (in mM): 125
NaCl, 25 NaHCO3, 10 glucose, 2.5 KCl, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, 0.5 L-ascorbic acid and 2 pyruvate (300–310 mOsm, bubbled with a 95%, 5% O2/CO2 gas mixture). Following lumbosacral laminectomy, the dura mater and pia-arachnoid membrane were carefully removed leaving intact dorsal roots. Para-saggital slices (400 μm) were prepared using a Vibratome 1000 Plus sectioning system (Vibratome) and stored in warm oxygenated (37°C) aCSF for 35 mins after which slices were kept at room temperature until required. Slices were transferred to a recording chamber and perfused at 2 mL/min with aCSF heated to 32°C. For experiments in which test compound was applied to the dorsal roots only, a recording chamber was used that consisted of two separate baths, each with their own perfusion inlets and outlets. The separate chambers were connected via tunnels that allowed the dorsal root to pass across and was then sealed using silicone grease. Both chambers were independently perfused at 2 mL/min with aCSF heated to 32°C. Whole-cell voltage clamp recordings were obtained from visually identified substantia gelatinosa neurons present in lamina II. ICS contained (in mM): 120 K gluconate, 10 NaCl, 2 MgCl2, 0.5 K2EGTA, 10 HEPES, 4 Na2ATP, 0.3 NaGTP, 20 biocytin (285–300 mOsm, pH adjusted to 7.2 with KOH).

Synaptic responses were evoked using a glass suction electrode to deliver a 400 μs stimulus of varying current amplitude (1 to 3.2 mA) applied every 15 sec via a digital stimulator (Digitimer Ltd, Hertfordshire, UK). Aδ-fiber EPSCs were distinguished from C-fiber EPSCs by the differences in latency to evoke an EPSC with C-fiber EPSC’s having longer latency than Aδ-fiber EPSC’s. Only monosynaptic evoked excitatory post synaptic currents (EPSC’s) were recorded. Perfusion to the spinal cord section also contained 50 μM picrotoxin and 50 μM strychnine to record only AMPA mediated EPSCs. Membrane signals were filtered at 2 kHz and sampled at 33 kHz.

**Synaptosome preparation and CGRP release assay**

Male adult CD1 mice were terminally euthanized as described above, the spinal column removed and submerged in ice-cold ACSF bubbled with a 95/5% O2/CO2. A lumbosacral laminectomy was performed and a segment of lumbar spinal cord was removed into a petri dish containing ice-cold pre-oxygenated aCSF. The dura mater, ventral and dorsal roots and the pia-arachnoid membrane were removed. A small segment of the spinal cord containing only the lumbar region (L4–L5) was dissected free, weighed and placed into ice-cold homogenization buffer composition (mM) 0.32 M sucrose, 10 mM HEPES (pH 7.4, bubbled with 100% O2). Spinal cord tissue was homogenized in buffer using 20 strokes of a glass/glass hand homogenizer. The crude homogenate was diluted with 3 mL of ice-cold homogenization buffer and centrifuged at 1000 x g for 5 mins at 4°C. Synaptosomes were isolated from the supernatant by an additional centrifugation at 20,000 x g for 2 mins at 4°C. The synaptosomal pellet was resuspended in 3 mL of Krebs buffer with composition (in mM): NaCl 118, KCl 2.4, CaCl2 2.4, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, glucose 10, pH 7.4 (bubbled with 95% O2 and 5% CO2). Protein quantification was performed on the synaptosomal solution using the Bradford colorimetric protein assay and bovine serum albumin standards. Samples were diluted to obtain a final concentration of 0.1 mg mL⁻¹ for CGRP evaluation and 100 μL samples of the synaptosomal solution was pipetted into eppendorfs. Synaptosomes were incubated at 37°C for 10 mins in the presence of test compounds or DMSO (1 μl total volume). Synaptosomes were stimulated using Veratridine 10 μM in the presence of Thiorphan (10 μM) and incubated for a further 10 mins at 37°C. Samples were then filtered using 0.22 μM filters and 100 μl of the filtrate was transferred to CGRP ELISA plate (Bertin Pharma) and assayed according to the manufactures instructions. Absorbance values were obtained using a microplate reader (Biorad model 680) at a wavelength of 415 nm.
Capsaicin flare

Adult Male C57Bl/6J Wild type (WT) and Na<sub>1.7</sub>Nav<sub>1.8cre</sub> mice were kept under a 12h light/dark cycle (lights on at 07:00) with food and water ad libitum. Up to 24h prior to dosing mice were shaved on both flanks and returned to their home cages. Subsequently, mice were grouped randomly and dosed orally with 1 or 10 mg/kg PF-05198007 or vehicle as appropriate (n = 8 per group). At 1h 15mins post dose mice were placed in an anesthetic chamber and anesthetized with a 5% isoflurane/O2 mixture. Anesthesia was maintained using a nose cone and animals transferred to a homeothermic blanket for the duration of the procedure. Laser Doppler flowmetry scans (MoorLDI apparatus, Moor Instruments Ltd, Axminster, United Kingdom) were taken from an area of the flank approximately 1.6cm<sup>2</sup>. Baseline scans were recorded for 35 mins, following which 50 μl of a 0.1% capsaicin (Sigma) dissolved in 100% ethanol was administered topically to the centre of the flank scan area using a 12mm polypropylene coated aluminium Finn chamber on Scanpor tape (Biodiagnostics Ltd, Worcestershire) for 10 min. Laser Doppler flowmetry scans were recorded for 55 mins post-removal of the Finn chamber. Laser Doppler flowmetry data was analyzed using proprietary software (MoorLDI, version 5.2). Baseline blood flow was calculated as the mean of the final 5 scans prior to capsaicin application.

Data analysis

Patch clamp electrophysiological data analyses were performed using either pClamp v10 software (Molecular Devices), PatchMaster software (HEKA) or Spike 2 v7 (CED). Action potential parameters (voltage threshold, peak amplitude, upstroke slope, width at threshold) were determined using a bespoke Spike 2 v7 analysis script courtesy of CED. Data are presented as mean ± standard error of the mean (SEM) Statistical comparisons were made using the paired Student’s t-test, one-way analysis of variance (ANOVA) with Bonferroni or Tukey’s post-tests, or ANOVA on ranks.

Drugs and reagents

PF-05089771 4-[2-(5-amino-1H-pyrazol-4-yl)-4-chlorophenoxy]-5-chloro-2-fluoro-N-(1,3-thiazol-4-yl)benzenesulfonamide and PF-05198007 [4-(2-(3-amino-1H-pyrazol-4-yl)-4-(trifluoromethyl)phenoxy)-5-chloro-2-fluoro-N-(thiazol-4-yl)benzenesulfonamide] were synthesized by Worldwide Medical Chemistry, Pfizer Worldwide Research & Development. Drugs were made up in 100% DMSO as a 10 mM stock, except for tetrodotoxin (TTX, Nanning Leaf Pharmaceuticals, Canada) which was dissolved in water. Final working concentrations were made on the day of the experiments in ECS.

Results

Discovery, potency and selectivity of PF-05089771 and PF-05198007

The identification of the clinical compound PF-05089771 and the closely related preclinical compound PF-05198007 as potent and selective inhibitors of the human Na<sub>1.7</sub> (hNa<sub>1.7</sub>) channel resulted from iterative structure activity relationship—based refinement of a series of novel arylsulfonamide Na<sub>1.7</sub> channel inhibitors. Arylsulfonamide inhibitors of Na<sub>1.7</sub> (as exemplified by PF-05089771, Fig 1A) were identified as inhibitors of partially inactivated hNa<sub>1.7</sub> channels by electrophysiological testing on the PatchXpress (Fig 1B and 1C) with a voltage protocol that set the holding potential to the empirically determined half-inactivation voltage for each cell (Fig 1B). For hNa<sub>1.7</sub> in HEK 293 cells the V<sub>1/2</sub> of inactivation ranged from -54 mV to -96 mV with a mean value of -77.7 mV (n = 291). State-dependence of block was likewise
Fig 1. PF-05089771 is a potent, state-dependent and selective inhibitor of Nav1.7.

A. Structure of PF-05089771 (4-(2-(3-amino-1H-pyrazol-4-yl)-4-chlorophenoxy)-5-chloro-2-fluoro-N-(thiazol-4-yl)benzenesulfonamide)

B. Representative PatchXpress current recordings illustrating the near-complete block following 300 nM PF-05089771 application to half-inactivated WT hNav1.7 channels (97% ± 3%, n = 10) which was partially reversed following a 5 min washout duration. In contrast there was minimal block following application of 300 nM PF-05089771 to resting WT hNav1.7 channels (5% ± 3%, n = 4). Inset: PatchXpress voltage protocols for half-inactivation (upper) and resting state (lower). For a full description of the voltage protocols see Methods.

C. Block of half-inactivated WT hNav1.7 channels (n = 6–22 per concentration) was nearly 1000-fold more potent than resting channels (n = 4–11 per concentration) (11

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assessed. In order to measure compound block at resting state, the original voltage protocol was modified to remove the conditioning step to the half-inactivation voltage, retaining all other aspects of the drug addition and voltage-control parameters (Fig 1B). Under these conditions the concentration-response relationship revealed an IC\(_{50}\) of 11 nM for half-inactivated channels and an IC\(_{50}\) of ~10 \(\mu\)M for resting channels, nearly 1000-fold less potent than seen with half-inactivated channels (Fig 1B and 1C).

Nav1.7 undergoes two independent alternative splicing events leading to the production of four splice isoforms of human Nav1.7 [19]. Implementing similar protocols as described above, PF-05089771 blocked all Nav1.7 splice variants with similar potency (Fig 1D, Table 1). Evaluation of the blockade of more distantly related channels, specifically, L-type calcium channels, KvLQT and hERG potassium channels, demonstrated that PF-05089771 is highly selective over these channels with IC\(_{50}\) values determined to be 10 \(\mu\)M. No blockade of the human and cynomolgus TRPV1 receptors was observed at tested concentrations of PF-05089771 up to 20 and 10 \(\mu\)M, respectively. (S1 Table).

To examine the molecular basis of the Nav1.7 interaction with this novel inhibitor we measured PF-05089771 inhibition of channels that were mutated to change (1) the natural molecular determinant for TTX sensitivity in domain 1 (hNav1.7 TTX-R, Y362S) [20], (2) local anesthetic binding residues (hNav1.7 LA Mut, F1737A/Y1744A) [21] and (3) a recently identified small molecule binding site in VSD4 (hNav1.7 M1,2,3 Y1537S/W1538R/D1586E) [22]. Data in Fig 1E show that the potency of PF-05089771 was not substantially affected (<3-fold) by mutation of either the TTX or local anesthetic binding sites hNav1.7 (IC\(_{50}\) 11 ± 1.3 nM); hNav1.7 TTX-R, (IC\(_{50}\) 21.6 ± 1.2 nM); hNav1.7 LA Mut, (IC\(_{50}\) 4.7 ± 1.2 nM). However, PF-05089771 potency was reduced ~100 fold (IC\(_{50}\) 1.15 ± 1.2 \(\mu\)M) by the hNav1.7 M1,2,3 mutation, indicating interaction of PF-05089771 with the domain 4 VSD (VSD4).

Based on differences in the cross species sequence homology at the VSD4 binding site for PF-05089771, the possibility of species-dependent changes in potency was investigated. The Nav1.7 orthologues were cloned from rat, mouse, dog and cynomologus macaque and each was heterologously expressed in HEK 293 cells to facilitate the cross species comparison. PF-05089771 was found to inhibit the sodium currents of mouse, dog and cynomologus macaque Nav1.7 with potency indistinguishable from that of the human channel (Table 2). However, the rat Nav1.7 potency was significantly decreased (IC\(_{50}\) 168 nM, 15.3-fold lower than human) compared to all other tested species, consistent with sequence divergence of the putative PF-

| 5N11L | 5A11S | 5A11L | 5N11S |
|-------|-------|-------|-------|
| IC\(_{50}\) (nM) | 33 | 20 | 16 | 11 |
| HillSlope | 1.4 | 0.8 | 0.9 | 1.0 |
| SEM | 1.1 | 1.2 | 1.2 | 1.1 |

Table 1. Potency of PF-05089771 across hNav1.7 splice variants.

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Nav1.7 contributes to action potential threshold and upstroke in small mDRG neurons

Previous studies have suggested that Na\textsubscript{v}1.7 contributes to electrogenesis by increasing the probability that the cell will reach action potential threshold [24] [20] [25]. To investigate this,
current-clamp recordings were made from acutely dissociated mDRG neurons in which a series of sequentially larger current steps was applied in order to accurately determine the action potential rheobase. PF-05198007 significantly increased rheobase (control: 173 ± 37 pA vs PF-05198007: 239 ± 47 pA, n = 7, p < 0.01, ANOVA, Fig 3), an effect that returned to the control level following washout (control: 173 ± 37 pA vs wash: 176 ± 35 pA, p > 0.05, ANOVA, Fig 3B and 3C). The effect of PF-05198007 on rheobase confirms that Nav1.7 is important in setting action potential threshold in small-diameter DRG neurons. Subsequently, the effect of PF-05198007 on additional parameters of the action potential waveform was investigated. In current-clamp mode, single action potentials were evoked from -70 mV at 10 second intervals by 20 ms current steps to approximately 1.3 x rheobase. The aim of this protocol was to provide reliable action potential generation whilst minimizing any contaminating influence of the
current step to the action potential waveform. In 6/13 small-diameter DRG neurons tested, PF-05198007 (30 nM) application resulted in action potential failure (Fig 4A) with the remaining 7 cells continued to exhibit action potential firing in the presence of PF-05198007. In the case of the latter, PF-05198007 impacted the action potential waveform whereby action potentials
appeared delayed and were of smaller amplitude relative to control (Fig 4B). The representative phase plot in Fig 4C reveals the extent of waveform attenuation in the presence of PF-05198007. In particular, there was a reduction in the rate of voltage change during both the upstroke and repolarization phases, coupled with a reduction in the peak amplitude. PF-05198007 application
resulted in a significant depolarization of action potential threshold (control: -27.8 ± 2.0 mV vs PF-05198007: -22.8 ± 1.6 mV, n = 10, p < 0.01) and a reduction in both spike amplitude (control: 43.0 ± 3.3 mV vs PF-05198007: 37.7 ± 3.9 mV, n = 10, p < 0.05) and upstroke slope (control: 137 ± 19 mV/ms vs PF-05198007: 86 ± 11 mV/ms, n = 10, p < 0.01, Fig 4D). In addition to confirming an important contribution to action potential threshold, these findings might suggest that Na\(_{\alpha,1.7}\) also contributes to the action potential upstroke phase. Alternatively, the changes in voltage trajectory that accompany a loss of threshold current might affect subsequent non-Na\(_{\alpha,1.7}\) sodium channel conductances so that the effects of PF-05198007 on amplitude and upstroke slope occur as an indirect consequence of Na\(_{\alpha,1.7}\) block. We tested this hypothesis by performing voltage-clamp experiments on mDRG neurons in which an action potential recorded from the cell under study was used as the command voltage (action potential voltage-clamp; Blair and Bean, 2002). The inward current component of the overlayed traces in Fig 4E (middle panel) shows an increased latency following PF-05198007 application accompanied by a decrease in the peak amplitude (in both cases p < 0.05, n = 7, Fig 4E, 4F and 4G). The subtraction of the residual current in the presence of PF-05198007 from the control current (Fig 4E lower panel) reveals an inward current that approximates to the Na\(_{\alpha,1.7}\) current flowing during the action potential. Comparison of this current time-course to that of the action potential waveform illustrates that the peak of the PF-05198007-sensitive current occurred towards the peak of the action potential rising phase, suggesting a role for Na\(_{\alpha,1.7}\) not only in defining threshold but also in the action potential rising phase.

The role of Na\(_{\alpha,1.7}\) in human DRG neurons

To test whether our findings from isolated mouse DRG neurons extend to those derived from humans, electrophysiological recordings were made from small-to-medium diameter (<60 μm) DRG neurons surgically resected from human donors (hDRG neurons) and maintained in cell culture for up to 9 days. A recent study has reported that the majority of this cell population exhibit hallmarks of nociceptors [16]. Fast-inactivating TTX-S sodium currents were observed in all hDRG neurons (n = 16, Fig 5A). For these currents, the midpoint of the voltage activation and inactivation was -32.2 ± 0.8 mV and -71 ± 1.7 mV respectively (Fig 5B) compared with -22.0 ± 1.7 mV and -73.4 ± 1.5 mV recorded from human Na\(_{\alpha,1.7}\) recombinantly expressed in HEK 293 cells (p < 0.05; n = 9–10, data not shown). To assess the contribution of Na\(_{\alpha,1.7}\) in human DRG neurons directly, TTX-S currents were again isolated using A-803467 and recorded before and after application of PF-05089771. At a selective concentration (30 nM), this Na\(_{\alpha,1.7}\) inhibitor blocked the majority of TTX-S current (75.5 ± 10.5%, n = 5, Fig 5D) whilst 100 nM resulted in complete block (Fig 5C and 5D). The IC\(_{50}\) for TTX-S current block in hDRG neurons was 8.4 ± 1.2 nM (n = 5–8 per concentration) vs 11 ± 1.2 nM for recombinantly expressed hNa\(_{\alpha,1.7}\). Together, these data confirm that Na\(_{\alpha,1.7}\) underlies the majority of TTX-S current in cultured nociceptive hDRG neurons. We next explored how Na\(_{\alpha,1.7}\) contributes to electrogensis in these cells. Fig 5E shows a voltage trace from a current-clamp recording in which we utilised the same current stimulus protocols as described for mDRG neurons (i.e. action potentials were evoked once every 10 seconds by 20 ms depolarizing current steps of amplitude just exceeding rheobase with the prestimulus V\(_{m}\) maintained at -70 mV). Under these recording conditions 30 nM PF-05089771 blocked action potentials in 3 out of 7 neurons and 100 nM resulted in action potential failure in 5 out of 8 cells tested (Fig 5E and 5F).

Contribution of Na\(_{\alpha,1.7}\) to nociceptor signalling in the spinal cord

Within mouse spinal cord slices, stimulation of dorsal roots evoked both A\(\delta\) and C fiber excitatory post-synaptic currents (EPSCs) in substantia gelatinosa (SG) neurons of the superficial
dorsal horn that were completely abolished by NBQX (5 μM) and TTX (500 nM; data not shown). Application of PF-05198007 to the whole preparation comprising spinal cord with attached dorsal root (30 nM; 20–30 mins) caused a significant inhibition of Aδ-fiber (by 46.9 ± 5.4%; n = 4, p < 0.05; ANOVA) and C-fiber (53.7 ± 10.1%; n = 8, p < 0.05; ANOVA, Fig 6A and 6B) evoked EPSC amplitudes. Under current-clamp conditions, electrical stimulation of the dorsal root evoked a single action potential in SG neurons. In the presence of 30 nM
PF-05198007, such evoked action potentials were abolished in 8 out of 9 neurons tested (Fig 6A, lower trace). Action potentials were also evoked by injection of depolarizing current into SG neurons. Application of PF-05198007 (30 nM) had no effects on action potentials evoked in this manner (n = 6; Fig 6C) suggesting a presynaptic site of action for PF-05198007.

Fig 6. PF-05198007 acts peripherally and centrally to influence neurotransmitter release. A. Upper: representative evoked EPSCs during control (blue) and after 30 mins PF-05198007 application (red). Lower: representative synaptically evoked action potential trace (blue) recorded in SG neurons of the dorsal horn following dorsal root stimulation. PF-05198007 (20 mins) abolished synaptically evoked action potentials (red). B. Example time course of EPSC block following PF-05198007 application to the whole preparation. C. Action potentials induced via current injection steps in SG neurons were not abolished by PF-05198007 (30 nM). Representative voltage traces are shown following current injection steps of -20, 0 and 50 pA before (blue traces) and after (red traces) PF-05198007 application. Line chart shows change in firing frequency (Hz) during control and after application of PF-05198007 for all neurons tested (n = 5, p > 0.05, paired t-test). D. Example time course of EPSC block following PF-05198007 application to the dorsal root alone. E. Representative EPSC traces and summary bar graph showing that the application of PF-05198007 (30 nM) to the dorsal root alone inhibited C-fibre mediated EPSCs and resulted in a significant conduction delay (n = 7, * p < 0.05; ANOVA on Ranks). F. PF-05198007 (30 nM; n = 15: 100 nM; n = 19) reduced veratridine evoked CGRP release in spinal cord synaptosomes. Reduction was compared with mexilitine (100 μM; n = 19), Ca²⁺ free conditions (n = 8) and TTX (500 nM; n = 6) (Data are shown ±SEM; * p < 0.05; ANOVA on Ranks).

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investigate the influence of axonally expressed Na$_{\text{v}}$1.7 we used a dual perfusion bath that allowed PF-05198007 to be applied to the dorsal root specifically, without exposure to the spinal cord itself. Application of PF-05198007 (30 nM) to the dorsal root inhibited C-fiber evoked EPSCs by 87 ± 9% (n = 7; Fig 6D and 6E), and caused a significant (p < 0.05) delay in the latency of the C-fiber mediated EPSC (Fig 6E) suggesting a role for Na$_{\text{v}}$1.7 in axonal action potential conduction.

To investigate the role of Na$_{\text{v}}$1.7 in neuropeptide release from primary afferent central terminals we investigated the actions of PF-05198007 on veratridine-induced CGRP release from synaptosomes prepared from L4/L5 spinal cord sections (Fig 6F). Both PF-05198007 (30 nM & 100 nM) and mexiletine (100 μM) significantly reduced veratridine evoked CGRP release (in all cases p < 0.05; ANOVA on Ranks).

**PF-05198007 reduces the capsaicin flare response in WT, but not Na$_{\text{v}}$1.7$^{\text{Nav1.8Cre}}$ mice**

To understand whether Na$_{\text{v}}$1.7 contributes to neuropeptide release at the free nerve endings of nociceptors in the skin, in addition to CGRP release from central primary afferent terminals, we investigated the effects of the PF-05198007 on the capsaicin flare response in vivo. For WT mice, application of capsaicin (0.1%) induced a sustained flare response (Fig 7). Oral pre-treatment with the Na$_{\text{v}}$1.7 inhibitor, PF-05198007 (1 and 10mg/kg) reduced the flare response to capsaicin for the duration of the observation period (55 mins; Vehicle; 4930 ± 751 versus 1 and

![Image](https://example.com/image.png)

**Fig 7. PF-05198007 reduces the capsaicin flare response in WT, but not Na$_{\text{v}}$1.7$^{\text{Nav1.8Cre}}$ mice.** A, B. Time-course plots showing the effects of PF-05198007 on skin blood flow measured before and after topical capsaicin application for WT (A) and Nav1.7$^{\text{Nav1.8Cre}}$ (B) mice (for each genotype, n = 8 per group). C, D. Corresponding summary bar graphs showing flare response measured as area under the curve for WT (C) and Nav1.7$^{\text{Nav1.8Cre}}$ (D) mice before and after PF-05198007 treatment. 1 mg/kg and 10 mg/kg PF-05198007 significantly reduced capsaicin-induced flare in WT mice (C, both 1 mg/kg and 10 mg/kg, p < 0.01, ANOVA) but had no effect in Na$_{\text{v}}$1.7$^{\text{Nav1.8Cre}}$ mice (D, both 1 mg/kg and 10 mg/kg, p > 0.05, ANOVA).

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10 mg/kg 1967 ± 472 and 2265 ± 382, respectively (n = 7), AUC, p < 0.05, Fig 7A). To confirm that Na\textsubscript{v}1.7 block underlied this effect, we repeated the experiment using Na\textsubscript{v}1.7\textsuperscript{Nav1.8 Cre} mice in which Na\textsubscript{v}1.7 is genetically deleted only in Na\textsubscript{v}1.8-positive nocicetors (Nasser et al., 2004). Application of capsaicin to Na\textsubscript{v}1.7\textsuperscript{Nav1.8 Cre} mice resulted in a reduced (AUC, p < 0.01 vs WT, Fig 7B)—albeit still statistically significant—flare response (AUC, p < 0.05 vs pre-capsaicin baseline). Importantly, the flare response in Na\textsubscript{v}1.7\textsuperscript{Nav1.8 Cre} mice was indistinguishable from the residual flare response in WT PF-05198007-treated mice. PF-05198007 (1, 10 mg/kg), had no effect on the flare response in Na\textsubscript{v}1.7\textsuperscript{Nav1.8 Cre} mice (for both doses, p > 0.05, Fig 7B) indicating that the Na\textsubscript{v}1.7 component of flare in WT mice was completely blocked by the selective Na\textsubscript{v}1.7 inhibitor whilst other mechanisms that contribute to flare remained unaffected. These data demonstrate a Na\textsubscript{v}1.7-mediated contribution to neuropeptide release in peripheral neurogenic flare.

Discussion

There recently has been intense interest in development of subtype-selective Na\textsubscript{v}1.7 inhibitors. This has arisen from the identification of gain-of-function mutations of Na\textsubscript{v}1.7 in individuals with rare pain syndromes IEM, PEPD and the more common disorder SFN, as well as the discovery of this channel’s link to channelopathy-associated congenital insensitivity to pain. Together, this constellation of gain-of-function and loss-of-function mutations demonstrates an essential and non-redundant role for Na\textsubscript{v}1.7 in pain [1]. Here we describe in vitro pharmacological properties of two subtype-selective Na\textsubscript{v}1.7 compounds: a clinical compound PF-05089771 and a structurally-related preclinical tool compound PF-05198007. Using these selective inhibitors we have confirmed a functional role of Na\textsubscript{v}1.7 that spans the sensory neuron axis—from the cutaneous free nerve endings, through to the DRG cell body, dorsal root and central terminals in the spinal cord.

Pharmacological properties of Na\textsubscript{v}1.7 subtype-selective compounds

The potency of PF-05089771 against human Na\textsubscript{v}1.7 channels stably expressed in HEK293 cells (IC\textsubscript{50}, 11 nM) was 10-fold and 16-fold higher than the closest sodium channel isoforms Na\textsubscript{v}1.2 and Na\textsubscript{v}1.6, respectively. Other sodium channel subtypes that have been shown to play important roles in the pain pathways and that are expressed in DRG neurons, Na\textsubscript{v}1.3 and Na\textsubscript{v}1.8 [26], are essentially unaffected by PF-05089771. A lack of validated heterologous expression system for Na\textsubscript{v}1.9 prohibited evaluation of the potency of PF-05089771 against this channel, which has been shown to play an important role in human pain conditions [27]. The potency and selectivity of PF-05089771 have been achieved by targeting a novel binding region of Na\textsubscript{v}1.7 proposed to be in the VSD4 [22,28]. The interaction of PF-05089771 with this novel binding site is further supported by the inability of mutations altering the local anesthetic and TTX binding sites in Na\textsubscript{v}1.7 to significantly alter the potency of PF-05089771 examined in vitro. The selectivity gained through interacting with the channel at this site is consistent with the variability of critical amino acids across sodium channel isoforms, supporting a modulatory effect of PF-05089771 on the VSD of Na\textsubscript{v}1.7. Finally, this is also the case for several species orthologues of Na\textsubscript{v}1.7 where PF-05089771 was found to preferentially inhibit human, mouse, cynomolgus monkey and dog Na\textsubscript{v}1.7 over rat Na\textsubscript{v}1.7. The rat orthologue possesses two variant residues at the VSD4 interaction site and uniquely displays a histidine residue at position M1, differentiating rat from the other species that were tested. Thus, the unprecedented selectivity profile achieved by PF-05089771 and the cross-species differences in Na\textsubscript{v}1.7 orthologue potency can be explained through the novel binding interaction of this class of compounds.
Nav1.7 dominates TTX-S current in small-diameter DRG neurons

Several lines of evidence have suggested that Nav1.7 is the predominant Na\textsubscript{v} TTX-S subtype expressed in C-fibres, while Nav1.7, Nav1.6 and Nav1.1 are the main TTX-S subtypes present in A-fibres \[29\] [11] [26] [30] [12] [1] [13]. Using RNA-Seq to analyse pooled small-diameter mouse DRG neurons, we also demonstrate that Nav1.7 is the major TTX-S subtype transcript in nociceptors. We found that high Nav1.7 expression translates at the functional level because the majority of the isolated TTX-S current in the cell body of small-diameter mouse and human DRG neurons was blocked by Nav1.7 inhibitors. This data is consistent with published work showing that TTX-S current in small-diameter DRG neurons from the Nav1.7\textsuperscript{+/−}Cre mouse is reduced by 70% compared to WT \[31\]. Taken together, these findings imply that Nav1.7 is likely to play a prominent role in nociceptor excitability.

The role of Nav1.7 in electrogenesis

Our electrophysiological studies demonstrate the important contribution of Nav1.7 to both mouse and human sensory neuron action potential firing. In particular, the effects of Nav1.7 inhibitors on mouse DRG rheobase provide the first pharmacological evidence using a small molecule selective inhibitor that native Nav1.7 acts as the threshold channel. These data are consistent with recorded changes in rheobase following Nav1.7 silencing in cultured nodose sensory neurons \[32\] and dynamic clamp analysis, using a Hodgkin-Huxley model of hNav1.7 gating \[31\]. Furthermore our observation of a reduction in action potential peak height and slope in the presence of PF-05198007 suggests a role for Nav1.7 in the upstroke of the action potential, in addition to the more accepted idea of Nav1.7 as a threshold channel. Indeed, using the action potential voltage-clamp technique \[33\] we found that Nav1.7 conducts during the upstroke as well as the during initiation stage of the action potential. Although Nav1.7 has been suggested to amplify the generator potential at the peripheral terminals thus acting as a threshold channel \[24\], we propose that another principal role for Nav1.7 in nociceptors is in action potential electrogenesis.

Central and peripheral role of Nav1.7 in nociceptors

The role of Nav1.7 in synaptic transmission within the spinal cord was addressed using patch-clamp recordings from dorsal horn SG neurons following stimulation of the dorsal root. Evoked EPSCs were significantly reduced in amplitude by PF-05198007 perfused over the entire spinal cord slice preparation. These findings agree with previous reports demonstrating reduced Substance P release from spinal cord slices of Na\textsubscript{v},1.7 knockout mice (Nav1.7 ablated in advillin-expressing neurons) following electrical stimulation of attached dorsal root \[34\] and the reduced frequency of spontaneous EPSCs in mouse lamina II neurons following the application of a Na\textsubscript{v},1.7 blocking antibody \[35\]. In addition, a central role of Na\textsubscript{v},1.7 has been demonstrated \textit{in vivo} where a reduction in pain behaviour in mice is observed following intrathecal injection of this antibody \[35\]. In this scenario, Na\textsubscript{v},1.7 block can occur at both dorsal root and outer laminae. Here, the specific contributions of Na\textsubscript{v},1.7 to both conduction and synaptic release in the spinal cord were further investigated using PF-5198007. Specifically, we observed a reduction in C-fibre mediated EPSC amplitudes recorded from SG neurons (with corresponding slowing of C-fibre conduction velocity) following local application of PF-05198007 to dorsal root, implicating Nav1.7 in axonal conduction. In support of this, significant block of A-and C-fibre action potentials in vagal sensory neurons has been reported following shRNA knockdown of Nav1.7 \[32\] and similar findings have been obtained from sciatic nerve in studies using toxins that selectively inhibit different Na\textsubscript{v} subtypes including Nav1.7 \[36\] [37]. Collectively, these data are consistent with a role for Na\textsubscript{v},1.7 in which it influences synaptic transmission.
transmission in the dorsal horn via actions either at a peripheral location (i.e. by facilitating or permitting conduction), or at a central location (i.e. by affecting CGRP/Substance P release from central terminals), or both.

TRPV1 expression has previously been shown to be limited to C-fibres in mouse [38] [39] [13]. Therefore, neurogenic flare induced by topical application of a non-noxious dose of capsaicin represents a C-fibre mediated mechanism in mouse. Using the Laser Doppler flowmetry to measure increased blood flow associated with peripherally mediated axon reflex vasodilation [40], capsaicin-induced neurogenic flare was suppressed by the Na\textsubscript{v}1.7 inhibitor PF-05198007 in wild-type mouse, but this inhibitor had no effect on the Na\textsubscript{v}1.7\textsuperscript{Nav1.8Cre} knock-out mouse at the same dose. These data suggest a key role of Na\textsubscript{v}1.7 in controlling neuropeptide release from peripheral terminals of peptidergic C-fibres and potentially a role in conduction associated with the axon reflex.

**Conclusion**

The data in the current study could be interpreted as supporting separate roles for Na\textsubscript{v}1.7 in neurogenic flare, conduction and presynaptic release. However, peptidergic terminals in the dorsal horn consist of en passant varicosities [41] giving rise to simple non-glomerular axo-dendritic synapses [42]. Voltage-gated sodium channels have also been identified in en passant varicosities of both posterior pituitary and mossy fibre terminals, where they have been suggested to contribute to action potential generation and conduction [43] [44]. Therefore, a simple unifying theory, which takes into account the clear role of Na\textsubscript{v}1.7 in action potential generation at multiple sites, is that Na\textsubscript{v}1.7 contributes to electrogenesis at, or close to, distal terminals in peripheral axon, in the dorsal root and at, or close to, neurotransmitter-releasing boutons of nociceptors. The development and availability of selective Na\textsubscript{v}1.7 inhibitors will aid future studies to delineate the interplay between Na\textsubscript{v}1.7 function and nociceptor signalling in both acute and chronic pain states.

**Supporting Information**

S1 Table. Off target activity of PF-5089771. (DOCX)

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**Author Contributions**

Conceived and designed the experiments: AJA ARB MLC JT ECP DP ZL MKP EBS. Performed the experiments: AJA ARB MLC JT MAM AW ECP RD DP ZL JAH MR MKP PJC. Analyzed the data: AJA ARB MLC JT MAM AW ECP RD DP ZL JAH MR MKP. Contributed reagents/
materials/analysis tools: AG BEM CW NAS RIS PAS JAH. Wrote the paper: AJA ARB MLC JT ME NC AR SDH DK SGW MKP RPB EBS.

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