Neuroscience

Equivalent excitability through different sodium channels and implications for the analgesic efficacy of selective drugs

Reviewed Preprint

Revised by authors after peer review.

About eLife's process

Reviewed preprint version 2
April 8, 2024 (this version)

Reviewed preprint version 1
November 1, 2023

Posted to preprint server
August 1, 2023

Sent for peer review
August 1, 2023

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Abstract

Nociceptive sensory neurons convey pain-related signals to the CNS using action potentials. Loss-of-function mutations in the voltage-gated sodium channel $Na_V1.7$ cause insensitivity to pain (presumably by reducing nociceptor excitability) but efforts to treat pain by inhibiting $Na_V1.7$ pharmacologically have largely failed. This may reflect the variable contribution of $Na_V1.7$ to nociceptor excitability. Contrary to claims that $Na_V1.7$ is necessary for nociceptors to initiate action potentials, we show that nociceptors can achieve equivalent excitability using different combinations of $Na_V1.3$, $Na_V1.7$, and $Na_V1.8$. Selectively blocking one of those Na_V subtypes reduces nociceptor excitability only if the other two subtypes are weakly expressed. For example, excitability relies on $Na_V1.8$ in acutely dissociated nociceptors but responsibility shifts to $Na_V1.7$ and $Na_V1.3$ by the fourth day in culture. A similar shift in Na_V dependence occurs in vivo after inflammation, impacting ability of the $Na_V1.7$-selective inhibitor PF-05089771 to reduce pain in behavioral tests. Flexible use of different Na_V subtypes exemplifies degeneracy – equivalent function using different components – and compromises the reliable modulation of nociceptor excitability by subtype-selective inhibitors. Identifying the dominant Na_V subtype to predict drug efficacy is not trivial. Degeneracy at the cellular level must be considered when choosing drug targets at the molecular level.

Significance statement

Nociceptors can achieve equivalent excitability using different sodium channel subtypes. The analgesic efficacy of subtype-selective drugs hinges on which subtype controls excitability. This contingency likely contributes to poor clinical outcomes.

eLife assessment

This **fundamental** study provides an unprecedented understanding of the roles of different combinations of NaV channel isoforms in nociceptors' excitability, with relevance for the design of better strategies targeting NaV channels to treat pain. Although the experimental combination of electrophysiological, modeling, imaging, molecular biology, and behavioral data is **convincing** and supports the major claims of the work, some results remain inconclusive and need to be strengthened by further evidence. The work may be of broad interest to scientists working on pain, drug development, neuronal excitability, and ion channels.

Introduction

Chronic pain affects between 11 and 40% of the population worldwide (1). Neuropathic pain, which is pain arising from damage to the somatosensory nervous system, is particularly hard to treat with only 30% of patients achieving moderate (≥30%) relief using available treatments (2, 3). New treatments are needed but a meagre 11% of analgesic drugs entering phase 1 trials are ultimately approved (4), triggering debate about why basic science discoveries are not yielding improved clinical outcomes (5). Suggested explanations include flaws in preclinical animal testing (6, 7) or clinical trial design (8) but biological explanations must also be considered. For example, degeneracy – the ability of a biological system to achieve equivalent function using different components (9) – complicates modulation of neuronal excitability by allowing changes in diverse ion channels to potentially subvert the therapeutic effect of a drug targeting a particular channel (10). These explanations are not mutually exclusive but degeneracy continues to receive little consideration.

Like most neurons, nociceptive sensory neurons (nociceptors) rely on spikes to transmit information. Their excitability is thus critical for relaying information to the CNS. Nociceptor excitability is increased in many pathological pain conditions and the resultant increase in afferent input drives chronic pain (11-13). Neuronal excitability depends on the complex interplay between diverse ion channels (14-16) but some channels seem to be particularly important for pain. For instance, loss- or gain-of-function mutations in the gene *SCN9A*, which encodes the voltage-gated sodium channel $Na_V1.7$, cause congenital insensitivity to pain (CIP) or painful neuropathies, respectively (17-19); for review see (20). In rodents, nociceptor-specific deletion of $Na_V1.7$ abolishes acute and inflammatory pain (21) but not neuropathic pain (22, 23). Neuropathic pain is blocked by deleting $Na_V1.7$ globally, including from sympathetic neurons (24, 25), though not if the deletion is induced in adulthood (26). Furthermore, loss-of-function mutations in $Na_V1.7$ do not consistently reduce nociceptor excitability (see Discussion) and the associated insensitivity to pain involves increased opioid signaling (27, 28), consistent with naloxone's ability to restore pain sensitivity in CIP patients (27, 29). These observations cast doubt on whether $Na_V1.7$ mutations produce CIP by reducing nociceptor excitability, pointing instead to a less direct mechanism that may be harder to reproduce pharmacologically.

Notwithstanding such reservations, several $Na_V1.7$-selective drugs have been developed (30-32) but none have yet passed phase 2 clinical trials (33-36). This has been attributed to poor target engagement (35, 37-39) yet prevention of the flare response by PF-05198007, a $Na_V1.7$-selective inhibitor, argues that at least some $Na_V1.7$ channels are blocked (40). But CIP patients exhibit a normal flare response (41), suggesting that their C fibers compensate for chronic loss of $Na_V1.7$ channels. Other $Na_V1.7$-selective inhibitors have struggled in phase 1 trials

because of autonomic side effects (e.g. (42)), as might be expected if those drugs block $Na_V1.7$ channels on sympathetic neurons, which is apparently necessary to prevent/reverse neuropathic pain (see above). But CIP patients exhibit normal autonomic function (17, 41), suggesting that their sympathetic neurons also compensate for chronic loss of $Na_V1.7$ channels. In those patients, might similar compensation occur in nociceptors and restore pain, only for that effect to be masked by enhanced opioid signaling (see above)? Descriptions of $Na_V1.7$ as "the" threshold channel imply that it is irreplaceable for nociceptor excitability, consistent on the surface with pain insensitivity due to loss-of-function mutations in $Na_V1.7$ but inconsistent with some past electrophysiological data (43, 44). Clarifying whether nociceptors rely on $Na_V1.7$ is an unresolved issue important for predicting the analgesic efficacy of $Na_V1.7$-selective inhibitors.

A serendipitous observation prompted us to reassess the role of $Na_V1.7$ in nociceptor excitability and the implications for drug efficacy. Specifically, we observed that tetrodotoxin (TTX), which inhibits $Na_V1.7$ and several other TTX-sensitive (TTX-S) sodium channels, had variable effects in nociceptors, dramatically reducing their excitability in some conditions but not in others. This variability reveals that nociceptors can achieve equivalent excitability using different sodium channel subtypes, some of which are TTX-resistant (TTX-R). We demonstrate that a $Na_V1.7$-selective inhibitor produces analgesia only when nociceptor excitability relies on $Na_V1.7$. Insofar as increasingly selective drugs are more likely to have their efficacy subverted by degeneracy, our results have profound yet underappreciated implications for target selection and drug development.

Results

Equivalent excitability can arise from different voltage-gated sodium (Na_V) channel subtypes

Small dorsal root ganglion (DRG) neurons (soma diameter <25 μm) tend to spike repetitively when depolarized by current injection (45). In our sample, most small neurons genetically identified as nociceptors (see Methods) spiked repetitively when tested 2-8 hours after dissociation (DIV0) or after 4-7 days in culture (DIV4-7), though the proportion of repetitively spiking neurons increased slightly over that interval (χ^2=4.51, p=0.034, chi-square test) (**Fig. 1A**). Strikingly, 100 nM TTX had no effect on the spiking pattern at DIV0 but converted all but one neuron to transient spiking at DIV4-7. Amongst neurons that spiked repetitively at baseline, TTX reduced the firing rate and increased rheobase only at DIV4-7 (**Fig. 1B**). TTX reduced spike height at DIV0 and DIV4-7, but more so at DIV4-7. There was a significant increase in capacitance and leak conductance density between DIV0 and DIV4-7, but no change in resting membrane potential (**Fig. 1C**). Normalizing leak conductance by capacitance (which increases over time because of neurite growth) disambiguates whether changes in input resistance reflect changes in cell size or membrane leakiness. Consistent with current clamp data, voltage clamp recordings showed that only a small fraction of sodium current is TTX-S at DIV0, whereas nearly all sodium current was blocked by TTX at DIV4-7 (**Fig. 1D**). Previous studies suggested that TTX-R channels play an important role in nociceptor excitability (46-48). Our initial results confirm this for DIV0 but show that their contribution diminishes after a few days in culture, with TTX-S channels becoming dominant by DIV4. Despite this reconfiguring of Na_V channels, excitability was remarkably stable, consistent with previous work showing little change in excitability after axotomy despite large (but evidently counterbalanced) changes in TTX-R and TTX-S currents (43, 49). We show later that similar changes develop in vivo following inflammation with consequences for drug efficacy assessed behaviorally (see **Fig. 8**), suggesting the Na_V channel reconfiguration described above is not a trivial epiphenomenon of culturing.

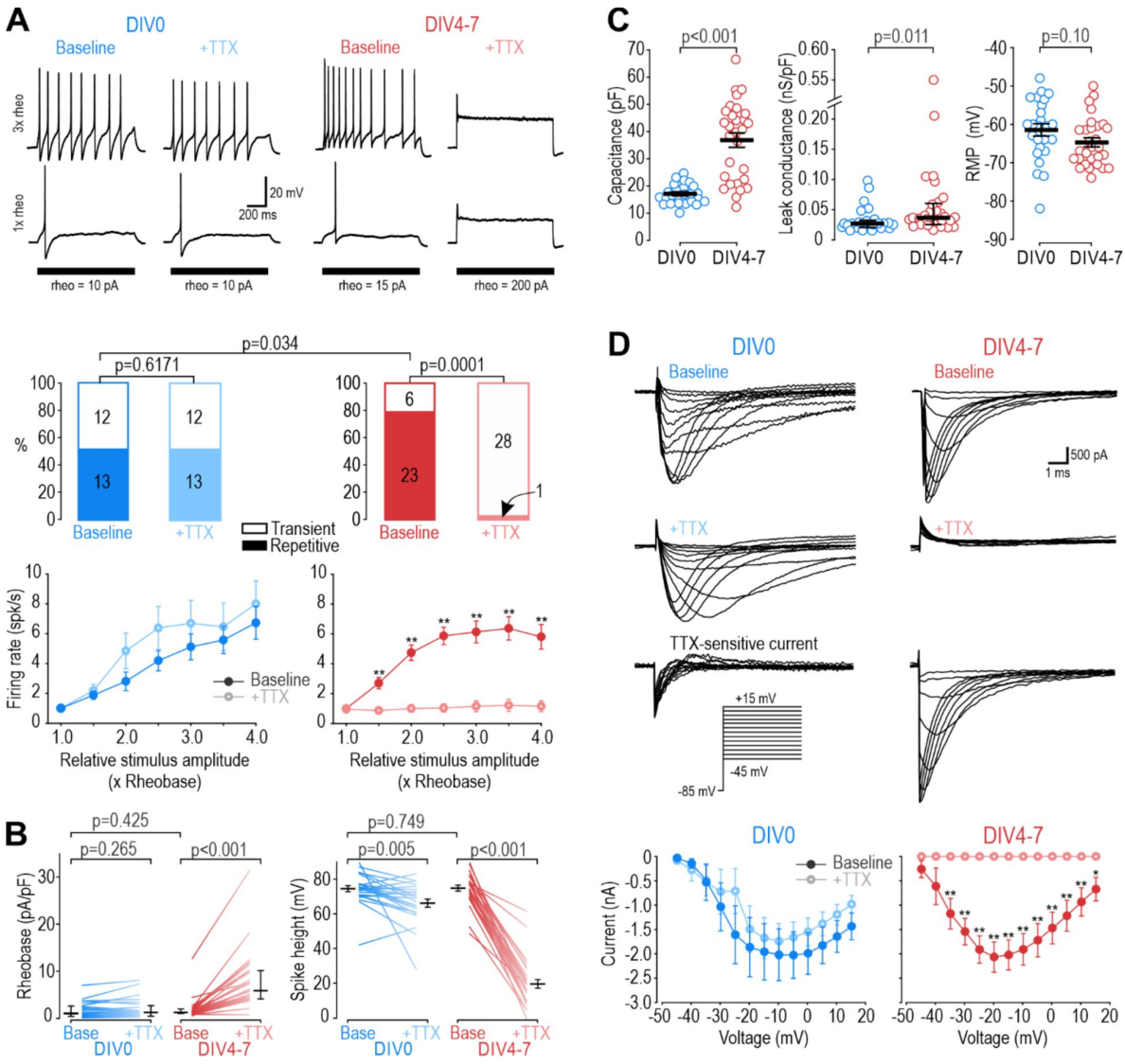

Figure 1.

Different Na_V subtypes produce equivalent excitability at different days in vitro (DIV).

(A) Representative responses of small DRG neurons to current injection at rheobase and 3x rheobase when tested on DIV0 (blue) or DIV4-7 (red) before (dark) and after (pale) bath application of 100 nM TTX. At DIV0, TTX did not alter spiking pattern (χ^2=0.25, p=0.617, McNemar test) or significantly reduce firing rate ($F_{1,72}$=1.527, p=0.24, two-way repeated measure (RM) ANOVA; n=13). At DIV4-7, TTX significantly altered spiking pattern, converting all but one neuron to transient spiking (χ^2=20.05, p<0.0001), and it significantly reduced firing rate ($F_{1,132}$=43.157, p<0.001, n=23). Only neurons with repetitive spiking at baseline are included in the firing rate plot. **(B)** At DIV0, TTX did not affect rheobase (Z_{24}=1.129, p=0.265, Wilcoxon rank test) but did reduce spike height (T_{24}=3.092, p=0.005, paired t-test). At DIV4-7, TTX increased rheobase (Z_{28}=4.681, p<0.001, Wilcoxon rank test) and dramatically reduced spike height (T_{28}=20.333, p<0.001, paired t-test). Notably, neurons at DIV0 and DIV4-7 did not differ in their baseline rheobase (U=316, p=0.425, Mann-Whitney test) or spike height (T_{52}=0.322, p=0.749, t-test). **(C)** Neurons at DIV0 and DIV4-7 differed in their total capacitance (T_{52}=6.728, p<0.001, t-test) and leak conductance density (U=216, p=0.011, Mann-Whitney test) but not in their resting membrane potential (T_{52}=1.668, p=0.101, t-test). **(D)** Sample voltage clamp recordings with command voltage stepped from -85 mV to +15 mV in 5 mV increments, before and after TTX. Sodium current was not significantly reduced by TTX at DIV0 ($F_{1,72}$=3.585, p=0.107, two-way RM ANOVA; n=7 neurons) but was completely abolished by TTX at DIV4-7 ($F_{1,108}$=33.526, p<0.001; n=10 neurons). *, p<0.05; **, p<0.01; Student-Newman-Keuls post-hoc tests in A and D. Traces labeled "TTX-sensitive current" represent the difference between current measured at baseline and after TTX, as determined by subtracting responses to the same voltage step under different pharmacological conditions.

Different Na_V channel subtypes control nociceptor excitability at DIV0 and DIV4-7

Next, we sought to identify the Na_V subtype responsible for repetitive spiking at each time point, starting with DIV0. Of the TTX-R Na_V channels expressed by nociceptors, $Na_V1.8$ has been implicated in repetitive spiking (47, 48). We measured sodium current in voltage clamp before and after applying the $Na_V1.8$-selective inhibitor PF-01247324 (PF-24) (50). At DIV0, 1 μM PF-24 abolished most of the sodium current (**Fig. 2A**). The PF-24-sensitive current had slow inactivation kinetics, like the TTX-R current and unlike the fast TTX-S current in **Figure 1D**, and consistent with previous descriptions of $Na_V1.8$ (51). A different $Na_v1.8$ antagonist, A-803467, had similar effects (**Fig. 2 – figure supplement 1**). In current clamp, PF-24 converted 7 of 8 repetitively spiking neurons to transient spiking and significantly reduced evoked spiking (**Fig. 2B**). It also increased rheobase and decreased spike height but did not affect resting membrane potential (**Fig. 2C**). PF-24 had negligible effects when tested at DIV4-7 (**Fig. 2 – figure supplement 2**). These results show that $Na_V1.8$ is the predominant Na_V subtype at DIV0 and is necessary for repetitive spiking at that time point. To test the sufficiency of $Na_V1.8$ to produce repetitive spiking, we tuned a single-compartment, conductance-based model neuron (see Methods) to reproduce DIV0 data described above. In this DIV0 model, inclusion of $Na_V1.8$ conductance was sufficient to generate repetitive spiking (**Fig. 2D** left). The necessity of $Na_V1.8$ for repetitive spiking at DIV0 was also recapitulated: 85% reduction in the $Na_V1.8$ conductance converted spiking from repetitive to transient (**Fig. 2D** and **Supplementary Table 1**).

Next, we sought to identify the Na_V subtype responsible for repetitive spiking at DIV4-7 using PF-05089771 (PF-71) to inhibit $Na_V1.7$ (40, 52) and ICA-121431 (ICA) to inhibit $Na_V1.1/1.3$ (53, 54). Since $Na_V1.1$ is expressed mostly in medium-diameter (A8) neurons (55) whereas $Na_V1.3$ is known to be upregulated in C fibers after injury (for review, see 56), we ascribe the ICA effect to blockade of $Na_V1.3$. In voltage clamp, sodium current was significantly reduced by 30 nM PF-71, and most of the remaining current was blocked by 1 μM ICA (**Fig. 3A**). In current clamp, each inhibitor (applied separately) converted a significant proportion of neurons to transient spiking and significantly reduced firing rate (**Fig. 3B**). This argues that $Na_V1.7$ and $Na_V1.3$ are both necessary for repetitive spiking at DIV4-7. Inhibiting $Na_V1.7$ increased rheobase, unlike inhibiting $Na_V1.3$, and caused a stronger reduction in spike height (**Fig. 3C**). Neither affected resting membrane potential. These results show that $Na_V1.7$ is the predominant Na_V subtype at DIV4-7, but not the only one. PF-71 had negligible effects when tested at DIV0 (**Fig. 3 – figure supplement 1**). We re-tuned our computational model to reproduce DIV4-7 data, with both $Na_V1.7$ and $Na_V1.3$ being required to produce repetitive spiking, meaning neither channel is individually sufficient (**Fig. 3D** and **Supplementary Table 1**). That said, inserting a higher density of either $Na_V1.7$ or $Na_V1.3$ could produce repetitive spiking in the absence of the other subtype (**Fig. 3 – figure supplement 2**), consistent with $Na_V1.7$ and $Na_V1.3$ also being interchangeable.

Acutely interchanging Na_V subtypes does not affect spiking pattern

The ability of $Na_V1.3$, $Na_V1.7$ and $Na_V1.8$ to each encourage repetitive spiking is seemingly inconsistent with the common view that each Na_V subtype contributes selectively to a different phase of the spike (for example, **Figure 3** in ref. 56). If $Na_V1.8$ were to activate exclusively at suprathreshold voltages, it could not initiate spikes and a different perithreshold-activating Na_V channel would be needed, which is clearly inconsistent with our data. To verify that $Na_V1.7$ and $Na_V1.8$ currents are each sufficient to produce repetitive spiking, we tested whether the $Na_V1.8$ current necessary for spiking in our DIV0 computer model could be replaced with $Na_V1.7$, and whether the $Na_V1.7$ current necessary for spiking in our DIV4-7 computer model could be replaced

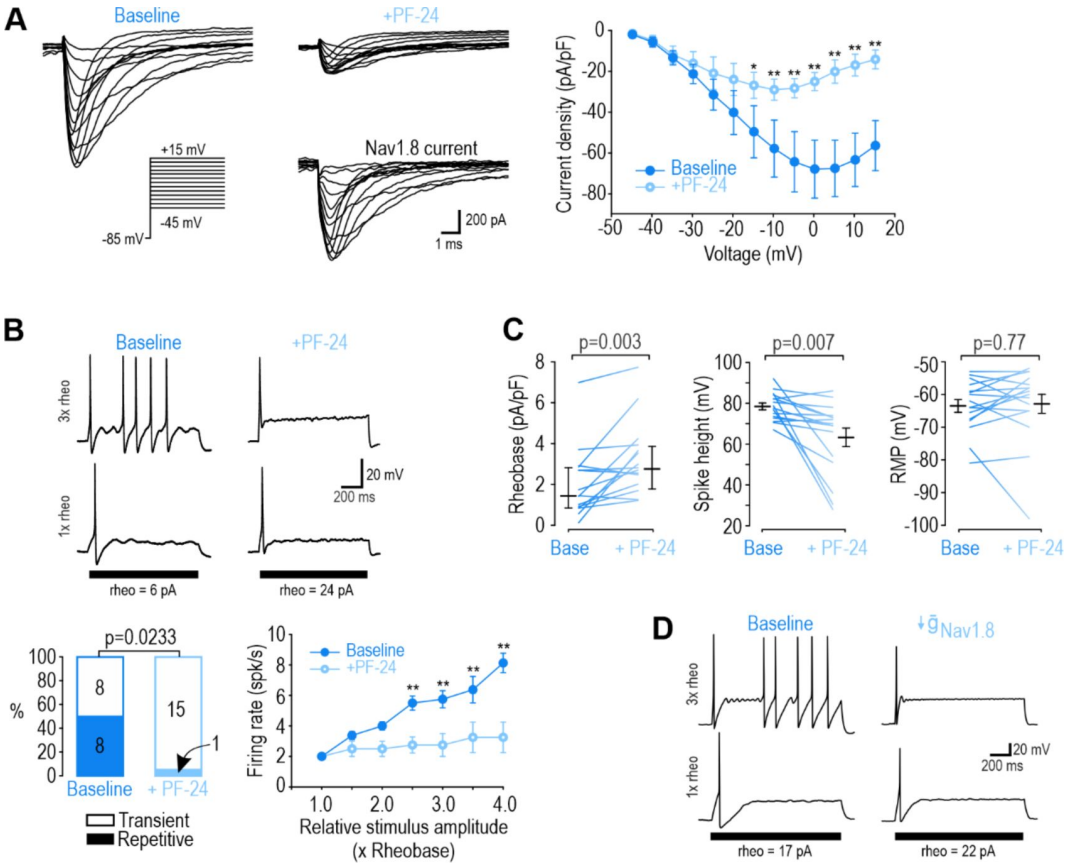

Figure 2.

$Na_V1.8$ is necessary for repetitive spiking at DIV0.

(A) Sample voltage clamp recordings show that sodium current was almost completely abolished by the $Na_V1.8$ inhibitor PF-24 (1 μM). Peak current was significantly reduced by PF-24 ($F_{1,72}$=12.651, p<0.012, two-way RM ANOVA; n=7). Traces labeled "$Na_V1.8$ current" represent the difference between current measured at baseline and after PF-24, as determined by subtraction. Another $Na_V1.8$ inhibitor, A-803467, had a similar effect (see **Fig. 2 – figure supplement 1**). **(B)** PF-24 significantly altered spiking pattern (χ^2=5.14, p=0.0233, McNemar test) and reduced firing rate ($F_{1,42}$=11.946, p=0.011, two-way RM ANOVA; n=8). **(C)** PF-24 significantly increased rheobase (Z_{15}=2.783, p=0.003, Wilcoxon rank test) and reduced spike height (T_{15}=3.151, p=0.007, paired t-test) but did not affect resting membrane potential (T_{15}=0.304, p=0.765, paired t-test). PF-24 had limited effects at DIV4-7 (**Fig 2** – figure supplement 2). **(D)** A computational model reproduced the effect of $Na_V1.8$ on spiking pattern (also see **Supplementary Table 1**). The PF-24 effect was simulated as a ~85% reduction in $Na_V1.8$ ($\bar{g}_{Nav1.8}$= 4mS/cm2). *, p<0.05; **; p<0.01; Student-Newman-Keuls post-hoc tests in A and B.

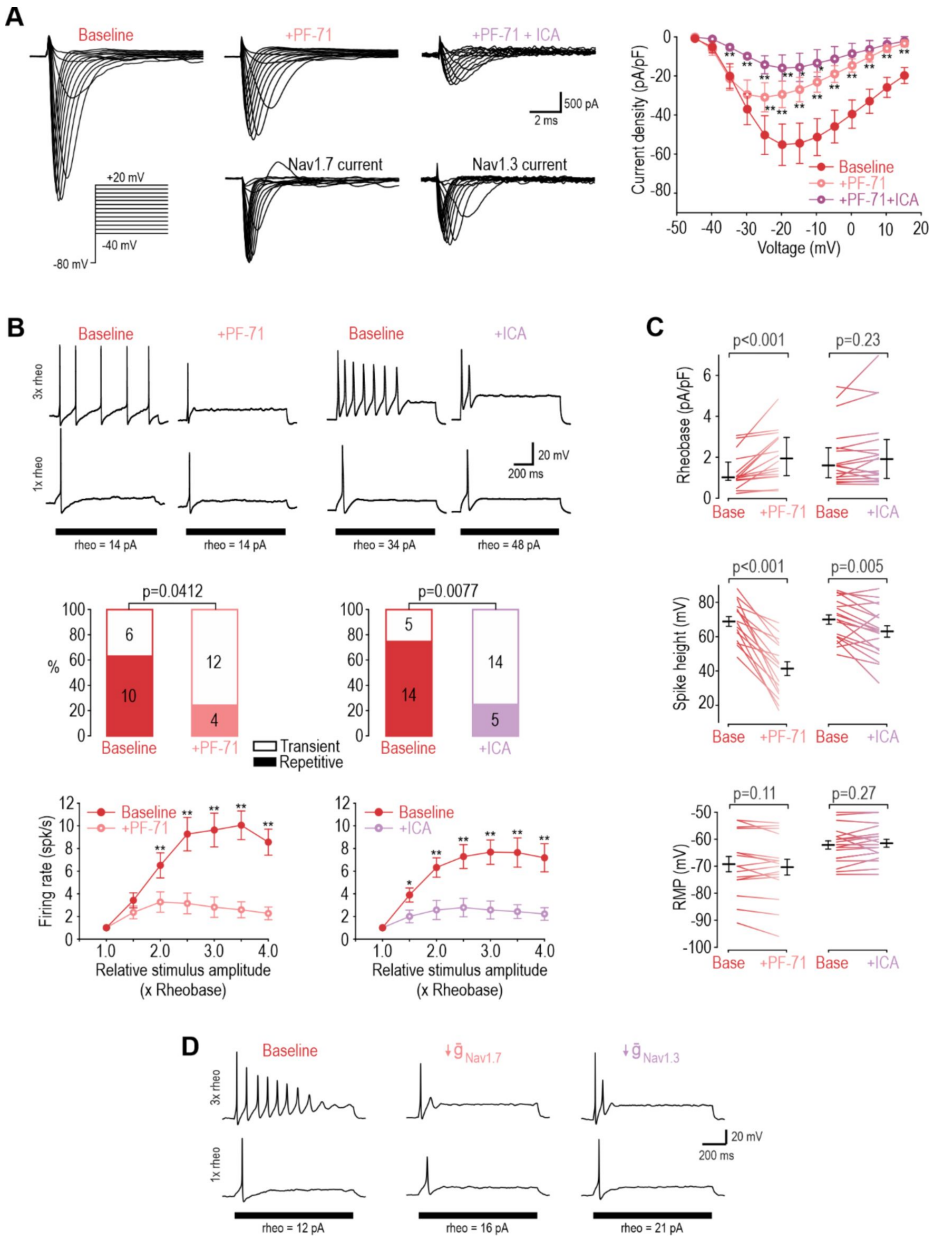

Figure 3.

$Na_V1.3$ and $Na_V1.7$ are necessary for repetitive spiking at DIV4-7.

(A) Sample voltage clamp recordings show that sodium current was reduced by the $Na_V1.7$ inhibitor PF-71 (30 nM) and by the $Na_V1.1/1.3$ inhibitor ICA (1 µM). Peak current was significantly reduced by PF-71 and ICA ($F_{2,192}$=26.361, p<0.001, two-way RM ANOVA; n=9). Traces labeled "$Na_V1.7$ current" and "$Na_V1.3$ current" represent the difference between current measured at baseline and after PF-72 and ICA, respectively, as determined by subtraction. **(B)** PF-71 and ICA both significantly altered spiking pattern (χ^2=4.17, p=0.041 and χ^2 =7.11, p=0.0077, respectively, McNemar tests) and significantly reduced firing rate ($F_{1,54}$=40.659, p<0.001, n=10 and $F_{1,78}$=35.156, p<0.001, n=14, respectively, two-way RM ANOVAs). **(C)** PF-71 significantly increased rheobase (Z_{18}=3.464, p<0.001, Wilcoxon rank test) and decreased spike height (T_{18}=7.946, p<0.001, paired t-test). ICA did not significantly alter rheobase (Z_{18}=1.248, p=0.225) but did reduce spike height (T_{18}=3.243, p=0.005). Neither drug affected resting membrane potential (T_{15}=1.681, p=0.113 for PF-71; T_{18}=-1.132, p=0.272 for ICA, paired t-test). PF-71 had negligible effects at DIV0 (**Fig. 3 – figure supplement 1**). **(D)** A computational model reproduced the combined effects of $Na_V1.3$ and $Na_V1.7$ on spiking pattern (also see **Supplementary Table 1** and **Fig. 3 – figure supplement 2**). PF-71 effect was simulated as a 70% reduction in $Na_V1.7$ ($\bar{g}_{Nav1.7}$= 10.5 mS/cm^2). ICA effect was simulated as a 90% reduction in $Na_V1.3$ ($\bar{g}_{Nav1.3}$'= 0.035 mS/cm^2). *, p<0.05; **, p<0.01; Student-Newman-Keuls post-hoc tests in A and B.

with $Na_V1.8$. In both cases, repetitive spiking was restored after inserting the alternate current (**Fig. 4A**). We then proceeded with equivalent experiments in real neurons, inhibiting $Na_V1.8$ with PF-24 on DIV0 or $Na_V1.7$ with PF-71 on DIV4-7, and then introducing the alternate channel virtually using dynamic clamp (see *Methods*). The replacement was successful in all neurons tested (**Fig. 4B**). Inserting virtual $Na_V1.8$ after inhibiting native $Na_V1.8$ also restored repetitive spiking, and likewise for $Na_V1.7$ (**Fig. 4 – figure supplement 1**), verifying that our virtual channels were equivalent to the native channels we aimed to replace. Apart from maximal conductance density, which was titrated in each neuron, all other parameters used for dynamic clamp were identical to simulations. The success of dynamic clamp experiments helps validate our computational models insofar as virtual $Na_V1.7$ and $Na_V1.8$ currents interacted appropriately with native currents to produce repetitive spiking in real neurons, the same way they interact with other simulated currents in the model neuron. Please note that tests reported In **Figure 4B** involve replacing a native channel with a different virtual channel (e.g. native $Na_V1.8$ replaced with virtual $Na_V1.7$) whereas tests reported in **Figure 4** – **figure supplement 1** involve replacing a native channel with the equivalent virtual channel (e.g. native $Na_V1.8$ replaced with virtual $Na_V1.8$); the former demonstrates that Na_V subtypes are interchangeable whereas the latter serves as a positive control.

With the model neurons thus validated, we used simulations to infer $Na_V1.7$ and $Na_V1.8$ currents during different phases of the spike (**Fig. 5A-D**). Since inward (depolarizing) current at voltages just below spike threshold is critical for spike initiation (57), we sought to identify which Na_V contributes to the subthreshold current. In the DIV0 model (**Fig. 5A,B**), subthreshold inward current was mediated mostly by $Na_V1.7$ during the first spike (left) but by $Na_V1.8$ during the second and all subsequent spikes (right). We interpret this to mean that the first spike is initiated using $Na_V1.7$ whereas all subsequent spikes are initiated using $Na_V1.8$. This is explained by the small $Na_V1.7$ conductance at DIV0 quickly inactivating during the first spike and remaining inactive during subsequent spikes (**Fig. 5 – figure supplement 1A**). This is consistent with experimental results, where repetitive spiking at DIV0 was unaffected by inhibiting $Na_V1.7$ (see **Fig. 1** and **Fig. 3 – figure supplement 1**) but was prevented by inhibiting $Na_V1.8$ (see **Fig. 2**). Inactivation of $Na_V1.7$ after the first spike was reflected by an increase in voltage threshold between the first and second spike in the model (**Fig. 5A**), which prompted us to check if the same increase was evident experimentally, which it was (**Fig. 5E**). This unanticipated simulation result also predicted that TTX should affect the voltage threshold of the first spike in DIV0 neurons despite not having other notable effects (see **Fig. 1**); as predicted, TTX caused a significant depolarizing shift in voltage threshold at DIV0 (**Fig. 5 – figure supplement 2**), further validating our model. In the DIV4-7 model (**Fig. 5C,D**), subthreshold inward current was mediated by $Na_V1.7$ and $Na_V1.3$ during the first spike (left) and during all subsequent spikes (right), with $Na_V1.8$ contributing little. Even though inactivation reduced $Na_V1.7$ and $Na_V1.3$ current after the first spike (**Fig. 5 – figure supplement 1B**), those channels nonetheless provided sufficient inward current to support repetitive spiking at DIV4-7. Inactivation at DIV4-7 was reflected, however, in a combination of higher threshold and lower spike overshoot for the second spike, both in the model (**Fig. 5C,D**) and in experiments (**Fig. 5E**).

These results demonstrate that each Na_V subtype does not contribute exclusively to a particular phase of the spike, and nor is each spike phase mediated exclusively by a particular Na_V subtype. Instead, each Na_V subtype contributes preferentially to a different spike phase depending on its voltage-dependency and on the conductance densities and inactivation status of other Na_V subtypes; for instance, $Na_V1.8$ is often said to activate only at suprathreshold voltages, during the upswing of the spike, after the spike is initiated by $Na_V1.7$; but if $Na_V1.7$ is absent or inactivated, voltage threshold shifts into the $Na_V1.8$ activation range, thus enabling $Na_V1.8$ to activate at voltages that are now subthreshold. Indeed, a subtype's contribution can shift rapidly (because of channel inactivation) or slowly (because of changes in conductance density; see below).

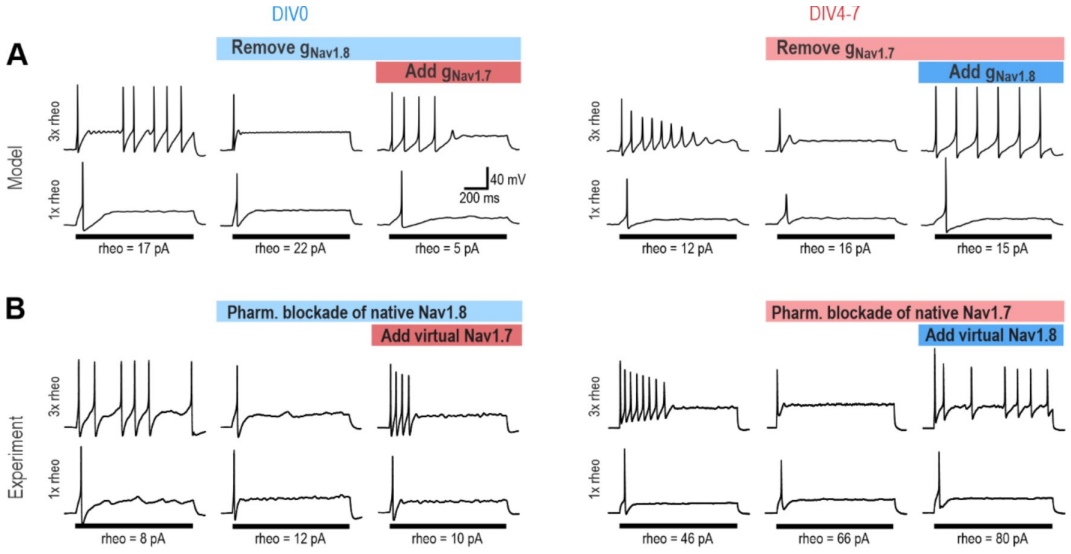

Figure 4.

$Na_V1.7$ and $Na_V1.8$ are each sufficient to produce repetitive spiking in DIV0 and DIV4-7 neurons.

(A) The computational model predicts that the $Na_V1.8$ conductance, which is "necessary" for repetitive spiking at DIV0 can, in principle, be replaced by $Na_V1.7$ (left), and vice versa at DIV4-7 (right). **(B)** Replacement experiments involved inhibiting native channels pharmacologically and then introducing virtual conductances using dynamic clamp. At DIV0 (left), inhibiting native $Na_V1.8$ (with PF-24) converted neurons to transient spiking, but introducing virtual $Na_V1.7$ reverted neurons to repetitive spiking (in 3 of 3 neurons tested). At DIV4-7, inhibiting native $Na_V1.7$ (with PF-71) converted the neuron to transient spiking, but introducing virtual $Na_V1.8$ reverted neurons to repetitive spiking (in 4 of 4 neurons tested). Repetitive spiking was likewise restored by replacing the blocked native channel with the corresponding virtual channel (**Fig. 4 – figure supplement 1**). Parameters for virtual channels were identical to simulations except for the maximal conductance density, which was titrated in each cell.

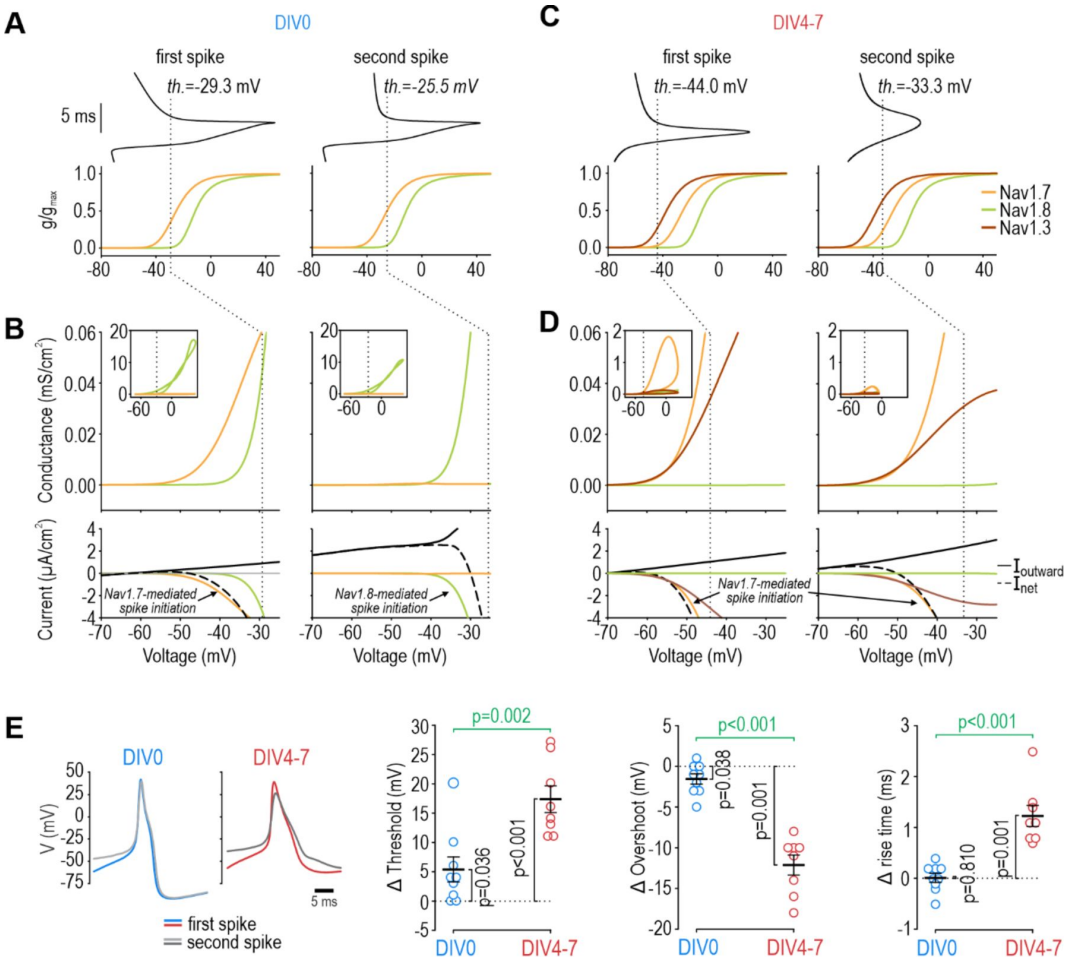

Figure 5.

Contribution of $Na_V1.7$ and $Na_V1.8$ to spike initiation in DIV0 and DIV4-7 neurons.

(A) Voltage (top) for first (left) and second (right) spikes in the DIV0 model aligned with voltage activation curves for each Na_V subtype (bottom). Dashed line shows voltage threshold (defined as V where dV/dt reaches 5 mV/ms). **(B)** Conductance plotted against voltage to create a phase portrait (top) showing Na_V conductance at different phases of the spike. Inset shows full voltage range; main graph zooms in on voltages near threshold. Bottom plots show current plotted over the same voltage range. Whereas $Na_V1.7$ (orange) mediated nearly all perithreshold inward current for the first spike, voltage threshold increased – because $Na_V1.7$ inactivated (**Fig. 5 – figure supplement 1**) – and $Na_V1.8$ (green) mediated nearly all perithreshold inward current for the second spike. The unexpected contribution of $Na_V1.7$ to the first spike correctly predicted that TTX increases voltage threshold in DIV0 neurons (**Fig. 5 – figure supplement 2**). (**C, D**) In the DIV4-7 model, $Na_V1.7$ (orange) and $Na_V1.3$ (maroon) contributed to initiation of all spikes whereas the contribution of $Na_V1.8$ was negligible (due entirely to its low expression level). **(E)** Sample experimental traces showing differences in the first (blue/red) and second (grey) spikes at DIV0 and DIV4-7. Plots summarize differences (Δ) in threshold, overshoot potential, and spike rise time between 1st and 2nd spikes during repetitive spiking evoked by current injection. At DIV0, the 1st and 2nd spikes differ significantly in their threshold (T_8=2.522, p=0.036, one-sample t-test) and overshoot (T_8=0.038, p=0.038) but not rise time (T_8=0.249, p=0.810). At DIV4-7, the 1st and 2nd spikes differ in all measures (threshold: T_7=7.613, p<0.001; overshoot: T_7=-9.849, p<0.001; rise time: T_7=5.979, p<0.001). Statistical results (green) show that differences between 1st and 2nd spike at DIV4-7 are significantly larger than differences at DIV0 (threshold: T_{15}=-3.847, p=0.002; overshoot: T_{15}=7.922, p<0.001; rise time: T_{15}=-5.617, p<0.001, unpaired t-tests), consistent with our computational model.

Changes in Na_V subtype expression between DIV0 and DIV4-7

Next, we sought to identify the basis for the slow shift in which Na_V subtype controls nociceptor excitability. **Figure 6A** shows mRNA levels for $Na_V1.7$ and $Na_V1.8$ relative to a housekeeping gene (left) and to each other (right). $Na_V1.7$ mRNA levels exceeded $Na_V1.8$ mRNA levels at both DIV0 and DIV7. Both decreased between DIV0 and DIV7, but $Na_V1.8$ more so, resulting in a significant decrease in the $Na_V1.8$:$Na_V1.7$ mRNA ratio. This pattern is consistent with the reduced role of $Na_V1.8$ at DIV4-7 but is inconsistent with the negligible role of $Na_V1.7$ at DIV0; specifically, we expected $Na_V1.7$ mRNA levels to increase between DIV0 and DIV7. Next, we investigated if functional changes were better reflected by changes in protein levels. Immunofluorescence for $Na_V1.8$ was higher than for $Na_V1.7$ at DIV0, and that ratio reversed at DIV7 (**Fig. 6B**), consistent with functional changes. Moreover, cercosporamide (10 μM), a potent inhibitor of the eukaryotic translation Initiation Factor 4E (eIF4E), significantly mitigated the decrease in $Na_V1.8$ immunofluorescence and the increase in $Na_V1.7$ immunofluorescence when applied to cultured neurons for 24 or 120 hours prior to measurements on DIV5 (**Fig. 6C**). Beyond showing that their mRNA levels do not correlate well with Na_V contributions to nociceptor excitability, reminiscent of some previous work (e.g. (58)), these results suggest that translational regulation is crucial, though membrane trafficking and other downstream processes likely also contribute (59, 60). Specifically, our results suggest that there is not enough pre-existing $Na_V1.7$ channels that trafficking those channels to the membrane can explain observed functional changes; instead, synthesis of new $Na_V1.7$ protein from existing $Na_V1.7$ mRNA is involved, but that does not rule out changes in how $Na_V1.7$ protein is handled or why Nav1.8 decreases. Further investigation is required to explore those mechanisms.

Analgesic efficacy of subtype-selective drugs depends on which Na_V controls nociceptor excitability

If a $Na_V1.7$-selective inhibitor mediates analgesia by modulating nociceptor excitability, its analgesic efficacy hinges on nociceptor excitability being controlled by $Na_V1.7$. Accordingly, we predicted that the $Na_V1.7$-selective inhibitor PF-71 would have little if any effect on paw withdrawal under normal conditions, when $Na_V1.8$ controls nociceptor excitability (**Fig. 2** and **Fig. 3 – figure supplement 1**), but would be effective if $Na_V1.7$ took over control. Inflammation increases $Na_V1.7$ channel trafficking and membrane expression (61-64). To test if inflammation increased $Na_V1.7$'s influence on nociceptor excitability, we recorded neurons acutely dissociated (DIV0) from DRGs of mice whose hind paw was injected with CFA three days prior. Sample traces in **Figure 7A** show that inflammation caused nociceptors to become much more variable in their reliance of specific Na_V subtypes, presumably because neurons innervating the inflamed paw experience greater effects of inflammation than neurons innervating other, non-inflamed sites. Specifically, application of PF-71 to inhibit $Na_V1.7$ converted 5 of 12 (42%) CFA neurons to transient spiking vs 0 of 9 (0%) of control neurons, which is a significantly higher proportion ($p = 0.0451$, Fisher exact test, **Fig. 7B**, **left**), whereas subsequent application of PF-24 to inhibit $Na_V1.8$ converted only 1 of 7 (14%) of the remaining repetitive spiking CFA neurons to transient spiking vs 7 of 8 (88%) of control neurons, which is a significantly lower proportion ($p = 0.001$). PF-71 also significantly affected resting membrane potential, rheobase, and spike height after CFA (**Fig. 7C**), unlike in control neurons (see **Fig. 3 – figure supplement 1**).

Results above confirm that $Na_V1.7$ takes on greater responsibility for nociceptor excitability after inflammation, which in turn predicts that PF-71 should reduce pain after inflammation but not under control conditions. As predicted, PF-71 significantly reduced thermal (**Fig. 8A**) and tactile (**Fig. 8B**) sensitivity in CFA-inflamed mice without having any effect in control mice. These results show that the inflammation-induced shift in Na_V subtype expression, despite being variable (see **Fig. 7**), is sufficient to cause a measurable change in drug efficacy assessed in vivo.

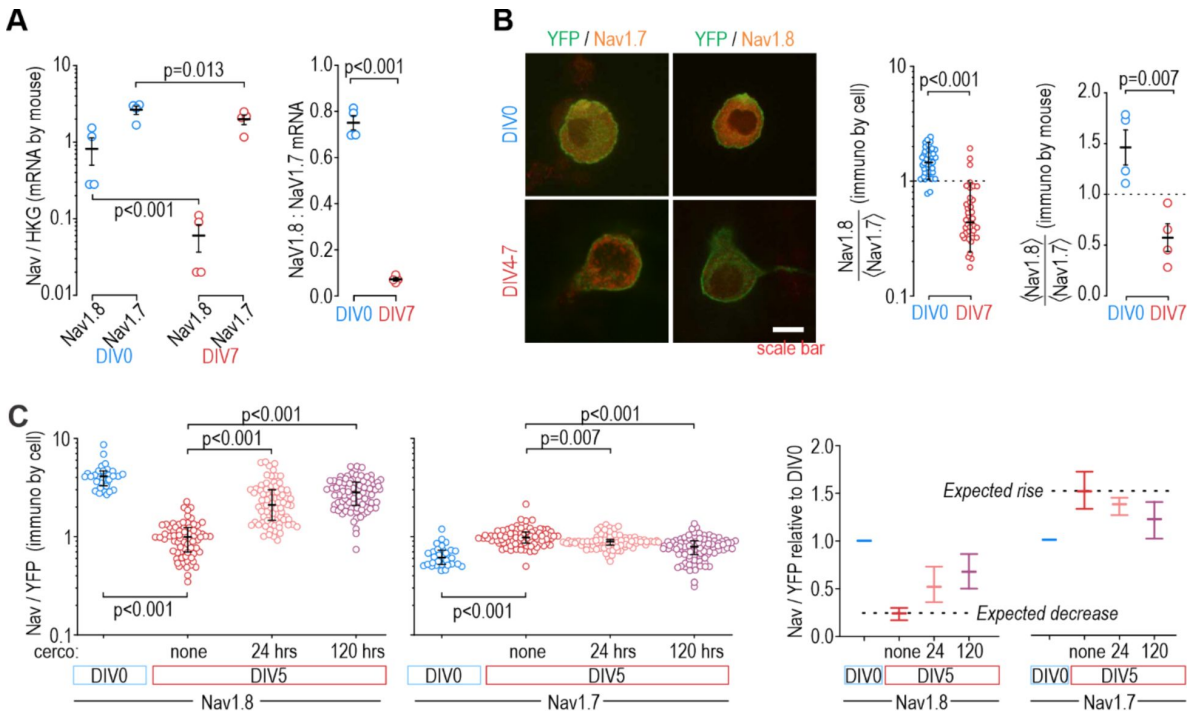

Figure 6.

Protein levels, but not mRNA, reflect functional contributions of Na_V subtypes at DIV0 and DIV7.

(A) Both $Na_V1.8$ and $Na_V1.7$ mRNA levels (relative to a housekeeping gene (HKG), see Methods) decreased significantly between DIV0 and DIV4-7 (factor 1: time, $F_{1,12}$=56.677, p<0.001, factor 2: subtype, $F_{1,12}$=17.952, p=0.001, two-way ANOVA and Student-Newman-Keuls post-hoc tests on log transformed data, n=4 mice per time point) but more so for $Na_V1.8$ than for $Na_V1.7$ (interaction: time x subtype, $F_{1,12}$= 11.455, p=0.005). The differential reduction yielded a significantly higher $Na_V1.8$:$Na_V1.7$ ratio at DIV0 than at DIV7 (T_6=21.375, p<0.001, unpaired t-test) but the increasing functional contribution of $Na_V1.7$ between DIV0 and DIV4-7 remains unaccounted for. **(B)** Immunoreactivity (IR) for $Na_V1.8$ protein exceeded $Na_V1.7$-IR at DIV0, but the opposite was true on DIV4-7, consistent with the functional contribution of each subtype. Na_V-IR was measured relative to YFP intensity in the same cell, and then each cell's $Na_V1.8$:YFP ratio was considered relative to the average $Na_V1.7$:YFP ratio in the co-processed coverslip (left) or average $Na_V1.8$:YFP ratio was considered relative to the average $Na_V1.7$:YFP ratio in the same animal (right). Ratios were >1 at DIV0 but decreased significantly at DIV4-7 (U=78, p<0.001, n=37 for DIV0, n=40 for DIV4-7, Mann-Whitney test (left) and T_6=4.046, p=0.007, unpaired t-test (right)). **(C)** Chronically applied cercosporamide (10 µM) mitigated changes in $Na_V1.8$- and $Na_V1.7$-IR at DIV5 ($Na_V1.8$: H_3=157.95, p<0.001; $Na_V1.7$: H_3=80.662, p<0.001; One-way ANOVA on ranks, Dunn's post-hoc tests, p<0.05 for all pairs). Data are summarized as median±quartile. Panel on the right shows data normalized to baseline (DIV0) to emphasize relative changes. N = 3 experiments.

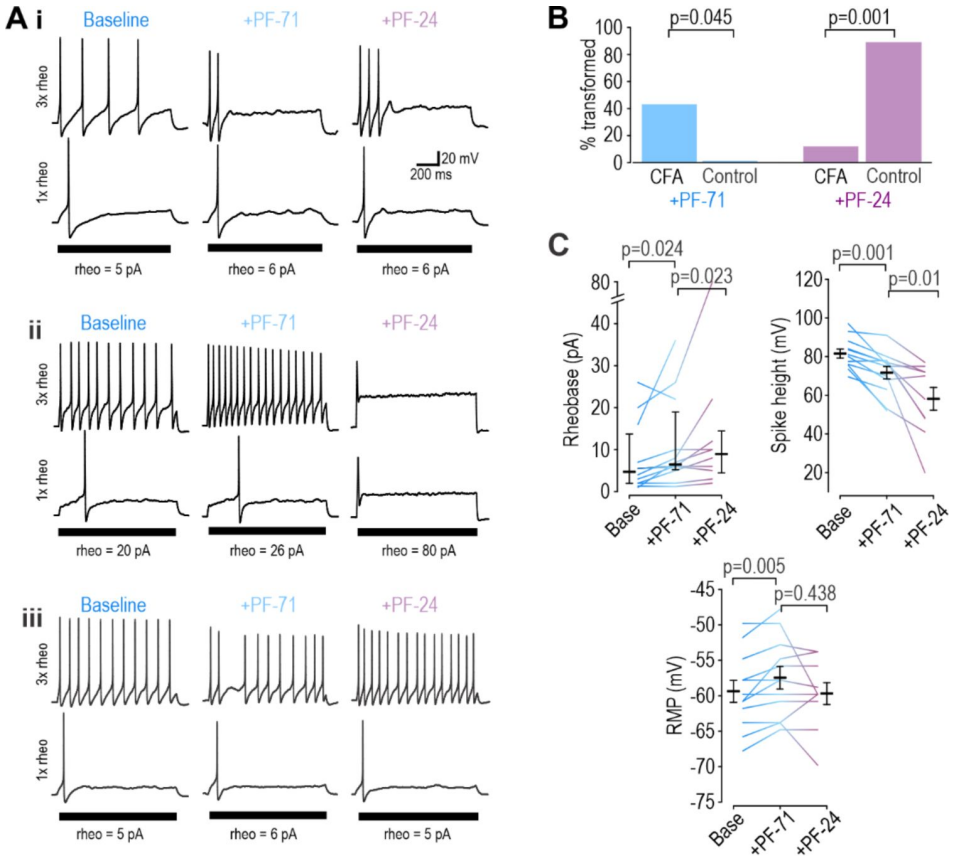

Figure 7.

Inflammation alters Na_V subtype contribution to nociceptor excitability.

(**A**) Sample responses in DIV0 neurons from mice injected with CFA three days earlier. In 12 cells tested, PF-71 converted 5 neurons to transient spiking (**i**), encouraged repetitive spiking in 4 neurons (**ii**), and had no effect in 3 neurons (**iii**), thus highlighting increased heterogeneity after CFA. (**B**) At DIV0, the effect of PF-71 differed significantly between CFA and control neurons, converting 42% (5 of 12) CFA neurons from repetitive to transient spiking vs 0% (0 of 9) control neurons (p=0.0451, Fisher Exact test). Applying PF-24 to neurons that continued to spike repetitively after PF-71 had little effect on CFA neurons, converting only 13% (1 of 7) of CFA neurons vs 88% (7 of 8) of control neurons (p=0.001, Fisher Exact test). Together these results argue that $Na_V1.7$ contributes more and $Na_V1.8$ contributes less to nociceptor excitability after inflammation. **(C)** At DIV0, PF-71 significantly increased resting membrane potential (T_{11}=-3.530, p=0.005, paired t-test) and rheobase (Z_{11}=2.186, p=0.024, Wilcoxon rank test), and significantly decreased spike height (T_{11}=4.413, p=0.001, paired t-test) in CFA neurons. Further addition of PF-24 significantly changed rheobase (Z_9=2.176, p=0.023, Wilcoxon rank test) and spike height (T_9=3.237, p=0.01, paired t-test) but did not affect resting membrane potential (T_9=1.049, p=0.321, paired t-test).

Consistent with this, epigenetic repression of $Na_V1.7$ prevents/reverses hypersensitivity in inflamed and neuropathic mice without causing hyposensitivity in naïve mice (65). This is unlike genetic deletion of $Na_V1.7$, which reduces thermal and tactile sensitivity in naïve mice (27), and with loss-of-function mutations in $Na_V1.7$ that abolish pain in humans (17). These inconsistencies rekindle concerns whether $Na_V1.7$ mutations, unlike pharmacological interventions, affect pain through mechanisms other than modulation of nociceptor excitability. Pharmacological reversal of hypersensitivity in chronic pain conditions (when $Na_V1.7$ is pathologically upregulated) without reducing normal nociceptive pain is clinically desirable, but this hinges on nociceptor hyperexcitability being $Na_V1.7$-dependent, which may be true of some but not all chronic pain conditions, or in only a subset of patients (66) (see Discussion).

Discussion

Our results show that nociceptors can achieve similar excitability using different Na_V channels. Whereas repetitive spiking depends on $Na_V1.8$ shortly after dissociation (**Fig. 2**) and presumably under normal conditions in vivo, responsibility shifts to $Na_V1.7$ and $Na_V1.3$ after a few days in vitro (**Fig. 3**). This is due to translationally regulated changes in Na_V expression (**Fig. 6**). Inflammation causes a similar shift in vivo (**Fig. 7**). Importantly, acutely inhibiting a particular Na_V is consequential (analgesic) only if that subtype is responsible for nociceptor excitability (**Fig. 8**). This may explain why $Na_V1.7$-selective drugs have not performed well in clinical trials (see Introduction) – because $Na_V1.7$ is not always necessary for nociceptor excitability depending on the expression level of $Na_V1.7$ and other Na_V subtypes. Faster processes like channel inactivation also affect their relative contribution (**Fig. 5**). These observations demonstrate the variable contribution of different Na_V subtypes to nociceptor excitability. When unaccounted for, such variability can lead to inconsistencies at the root of poor reproducibility and translatability.

Although we have focussed here on Na_V channels, numerous other channels are likely to change during culturing and in response to subtler, more clinically relevant in vivo insults like inflammation. Those changes occur through diverse mechanisms. Our results argue that transcriptional changes in $Na_V1.7$ do not account for its upregulation between DIV0 and DIV4-7, but transcriptional changes in other genes might nonetheless be important. And though our data implicate translational regulation, much more work is needed to work out the details. That work should consider the other ion channels and associated proteins (e.g. beta subunits) that interact with Na_V channels, either physically or via mutual effects on membrane potential. In a degenerate system, one should ideally consider all the components contributing to the process of interest, but perfect is the enemy of good, which is to say that degeneracy should still be considered even with incomplete understanding of the system. Indeed, the interchangeability of Na_V subtypes demonstrated here may help explain why subtype-specific drugs are not reliably effective again pain, even if myriad other still unidentified changes are also taking place.

Contrary to the view that certain ion channels are uniquely responsible for certain aspects of neuronal function, neurons use diverse ion channel combinations to achieve similar function (67, 68). This degeneracy is crucial for enabling excitability and other aspects of neuron physiology to be homeostatically regulated by adjusting ion channels in response to perturbations (69-71). Degeneracy also enables pathological changes in different ion channels to produce equivalent hyperexcitability (72). This is important insofar as similar excitability may belie differences in the underlying ion channels – differences that may render a neuron susceptible or impervious to a drug depending on the functional necessity of the targeted ion channel in that neuron. This is precisely what our data demonstrate in nociceptors. Similar observations have been made in substantia nigra neurons, whose pacemaker activity can be mediated by Na_V channels or by voltage-gated calcium channels, meaning TTX may or may not block their spiking (72, 73). Similar interchangeability is evident for the burst firing of Purkinje neurons (74).

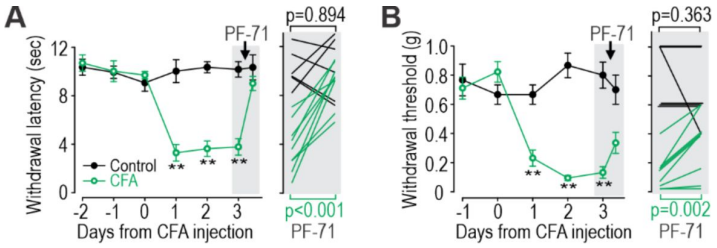

Figure 8.

Inflammation-induced change in Na_V subtype contribution impacts analgesic efficacy of PF-71.

(A) CFA significantly increased thermal sensitivity ($F_{5,65}$=19.556, p<0.001, two-way RM ANOVA). PF-71 significantly decreased thermal sensitivity in mice injected three days prior with CFA (T_8=-7.296, p<0.001; paired t-test) but had no effect in naïve mice (T_5=-0.141, p=0.894). **(B)** CFA significantly increased mechanical sensitivity ($F_{4,52}$=16.786, p<0.001). PF-71 significantly decreased tactile sensitivity in mice injected three days prior with CFA (T_8=-4.341, p=0.002) but had no effect in naive mice (T_5=1.000, p=0.363). Insets in both panels show values for each animal before and 2 hours after PF-71 injection. *, p<0.05; **, p<0.01; Student-Newman-Keuls post-hoc tests.

Degeneracy also exists at the circuit level (75, 76), where it allows differences in the intrinsic excitability of component neurons to be offset (and effectively hidden) by differences in synaptic weights (77). Relevant for pain processing, the spinal dorsal horn circuit can achieve similar output using different synaptic weight combinations (78); specific neuron types may have a greater or lesser impact on circuit function depending on those weights. In effect, degeneracy introduces contingencies. The role of any ion channel in a neuron (or any neuron in a circuit) depends on the other ion channels in that neuron (or the synaptic connections with other neurons in the circuit). Because of such contingencies, a drug may engage its target without producing the intended cellular, circuit or clinical effect. Indeed, different combinations of $GABA_A$ receptor activation and chloride driving force can produce equivalent synaptic inhibition (79), but when inhibition is incompensably compromised, the underlying cause necessitates different interventions (80). By this logic, if upregulation of $Na_V1.7$ is responsible for nociceptor hyperexcitability after nerve injury or inflammation, $Na_V1.7$ is an ideal target since "normal" neurons (not reliant on $Na_V1.7$) would be spared the effects of a $Na_V1.7$-selective drug, but the long-term efficacy of such a drug hinges on hyperexcitability remaining $Na_V1.7$-dependent, which cannot be assumed (10). Furthermore, if myelinated afferents (which express minimal $Na_V1.7$ (81)) are responsible for mechanical allodynia under neuropathic conditions (82-85), then $Na_V1.7$-selective drugs should not be expected to alleviate that symptom, which evidently they do not (26), at least not through a direct mechanism. Indeed, ablating nociceptors abolishes acute and inflammatory pain but not neuropathic pain (23, 86). Pathological pain being mediated by more than one afferent type is another example of circuit-level degeneracy.

To be interchangeable, Na_V subtypes must functionally overlap (87, 88). Indeed, $Na_V1.8$ and $Na_V1.7$ are similar but not identical in their gating properties; for example, their voltage-dependencies partially overlap but the activation curve for $Na_V1.8$ is right-shifted compared to $Na_V1.7$ (89). Consequently, $Na_V1.7$ activates at voltages near threshold whereas $Na_V1.8$ tends to activate at suprathreshold voltages, during initiation and upstroke of the spike, respectively (34, 56). But that separation is not absolute. We found that $Na_V1.7$ contributes to initiation of the first spike in DIV0 neurons, but because it inactivates more readily than $Na_V1.8$, initiation of all subsequent spikes depends on $Na_V1.8$ (see **Fig. 5**), which activates at perithreshold voltages because voltage threshold is high (depolarized) in the absence of $Na_V1.7$. At DIV4-7, $Na_V1.7$ still inactivates (which causes voltage threshold to rise) but, because of its higher density, continues to produce enough inward current to continue to initiate later spikes. The activation pattern we report for the first spike at DIV0 is consistent with Blair and Bean (90) who quantified the contribution of different Na_V channels by recording pharmacologically isolated currents while varying the holding potential according to the spike waveform. Our results go further in showing how responsibilities shift across different spikes within a train (because of differential Na_V inactivation) and across conditions (because of changes in Na_V expression). Although we focused on Na_V channels in this study, other ion channels are likely also undergoing changes; indeed, changes in the AHP shape between DIV0 and DIV 4-7 (see **Figure 1A**) point to changes in potassium channels. All neurons presumably regulate their excitability in a degenerate manner but likely do so by adjusting different sets of ion channels. Even within a given neuron type, the identify of adjusted ion channels may depend on the nature of the perturbation or on a multitude of other conditions.

With respect to reproducibility, labs testing nociceptors after different times in vitro would be expected to reach contradictory conclusions about the relative importance of a given Na_V subtype. Likewise, a testing protocol focusing on single spikes (the equivalent to the first spike in a train) would yield different results from one that considers repetitive spiking. Along the same lines, voltage clamp protocols that deliberately hold membrane potential at unnaturally hyperpolarized voltages to relieve inactivation before stepping up the voltage can give a misleading impression of how much a Na_V subtypes contributes under natural conditions (i.e. with natural levels of inactivation). Such discrepancies might be chalked up to irreproducibility if the consequences of those methodological differences are not appreciated, especially if one overlooks how degeneracy

allows responsibilities to shift between ion channels. Indeed, the pain literature is replete with apparent inconsistencies. We would argue that most of those studies are correct, but only under limited conditions. Failure to identify and report those conditions (contingencies) represents a huge impediment to translation. A recent review of degeneracy in epilepsy (91) reveals many similarities with chronic pain. Observations that effective antiseizure medications often act on multiple targets and that patients with the same type of epilepsy respond heterogeneosly to a given treatment offer circumstantial evidence of degeneracy, but more deliberate testing is required to quantify degeneracy and its impact.

In summary, our results show that nociceptors can achieve equivalent excitability using different Na_V subtypes. The importance of a given subtype can shift on long and short timescales, yielding results that are seemingly inconsistent. By elucidating those shifting responsibilities, our results highlight the degenerate nature of nociceptor excitability and its functional implications. Degeneracy makes it impossible to claim without reservation that a particular Na_V subtype is uniquely responsible for pathological pain. Greater appreciation of degeneracy's implications would prompt better experimental design, more cautious interpretation, and, ultimately, improved translation.

Materials and methods

Animals

All animal procedures were approved by the Animal Care Committee at The Hospital for Sick Children (protocol #53451) and were conducted in accordance with guidelines from the Canadian Council on Animal Care. We used the Cre-*lox*P recombinase system to generate mice that express ChR2-eYFP in $Na_V1.8$-expressing neurons. Mice were obtained by crossing homozygous Ai32 mice (B6.Cg-Gt(ROSA)26Sortm32(CAG-COP4*H134R/EYFP)Hze/J) from Jax (#012569), which express ChR2(H134R)-eYFP in the presence of Cre recombinase, with $Na_V1.8$-Cre mice (Tg(Scn10a-cre)1Rkun), which express Cre recombinase in $Na_v1.8$-expressing neurons (kindly provided by Rohini Kuner). These neurons are primarily nociceptive and thermoreceptive (92). The $Na_V1.8$ promoter leads to transgene expression in >90% of neurons expressing markers of nociceptors (21). To ensure that our transgenic mice were typical of wild-type mice with the same background (C57BL/6j), experiments reported in **Fig. 1** were repeated in both genotypes for comparison. There was no effect of genotype on rheobase, spike height, input resistance, or spiking pattern, nor was there any significant interaction between genotype and effects of TTX except for spike height at DIV4-7, where TTX had a marginally larger effect in wild-type mice (Two-way ANOVA, $F_{1,54}$=4.968, p=0.03, see source data file); therefore, we pooled the data for **Fig.1**. Having verified that our foundational observations held across different genotypes, we used transgenic mice for all subsequent experiments in order to identify eYFP-expressing nociceptors for patching, collection, or imaging.

Dorsal root ganglia neuron cultures

All key reagents are listed in **Supplementary Table 2**. Methods for primary DRG culture have been described previously (93). Briefly, adult mice (> 7 week-old) were anaesthetised with isoflurane and perfused intracardiacally with cold HBBS (without Ca and Mg, LifeTech 14170112) supplemented with (in mM) 15 HEPES, 28 Glucose, 111 sucrose, and pH adjusted with NaOH to 7.3-7.4; osmolarity 319-321. Lumbar dorsal root ganglia (DRGs) were extracted (L2-5, except for CFA-inflamed mice, in which we only took L4), digested with papain (Worthington Biochemical Corp.) and collagenase (Worthington Biochemical Corp.)/dispase II (Sigma), and mechanically dissociated by trituration before being plated onto poly-D lysine-coated coverslips and incubated in Neurobasal™ media (Gibco 21103-049) supplemented with 1 % fetal bovine serum (FBS), B-27™ supplement (Thermo Fisher 17504-044) and 0.5mM L-Glutamine (Gibco 25030-081) for an initial period of 2 hours. After this, media was changed to maintenance media (same as plating media but

without FBS) and cells were maintained in a 5% CO_2 incubator at 37°C. Media was changed every 3-4 days thereafter. Neurons were recorded at two time points after plating: 2-8 hours (referred to as DIV0) or 4-7 days (referred to as DIV4-7). Neurons were tested at intermediate time points (DIV1-3) in the exploratory phase of our study but cellular heterogeneity prevented a clear picture of their TTX-sensitivity, presumably because different neurons shift from $Na_V1.8$ to $Na_V1.7$ at different rates. By DIV4, TTX sensitivity had stabilized, and so recordings from DIV4-7 were pooled for comparison with recordings on DIV0. When tests were conducted on a specific day within the DIV4-7 range, the specific DIV is reported but should be considered representative of the DIV4-7 range. Additional experiments are required to determine the exact time course of the Na_V switch and its mechanistic basis; to mitigate effects of cellular heterogeneity, this would ideally involve techniques that allow longitudinal measurements in the same cell.

Electrophysiology

Coverslips with cultured neurons were transferred from the incubator to a recording chamber perfused with artificial cerebrospinal fluid containing (in mM): 126 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $MgCl_2$, and 10 glucose, bubbled with carbogen (5% CO_2:95% O_2) at room temperature. Neurons were visualized with gradient contrast optics on a Zeiss AxioExaminer microscope using a 40x, 0.75 NA water immersion objective (N-Achroplan, Zeiss) and IR-1000 Infrared CCD camera (Dage-MTI). YFP expression was visualized by epifluorescence (X-Cite, Excelitas) using a Zeiss filter set (46HE). A long-pass filter (OG590) was positioned in the transmitted light path to avoid activating ChR2 while patching. No optogenetic testing was performed as part of this study. Cells expressing YFP and with a soma diameter <25 μm were targeted for whole cell recording using pipettes (~5 MΩ resistance) pulled from borosilicate glass (WPI).

For current clamp recordings, pipettes were filled with intracellular solution containing (in Mm): 140 K-gluconate, 2 $MgCl_2$, 10 HEPES, 0.2 EGTA, 3.8 Na-ATP and 0.4 Na-GTP with pH adjusted to 7.3 with KOH; osmolarity was ~300 mOsm. A liquid junction potential correction of 15 mV was applied to all reported voltages. Series resistance was compensated to >70%. Signals were amplified with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, USA), low-pass filtered at 2 kHz, digitized with a Power1401 A/D device (Cambridge Electric Design, Cambridge, UK), and recorded at 10 kHz using CED software Signal version 6. After the natural resting membrane potential was noted, neurons were adjusted to -70 mV using continuous current injection in current clamp mode. Action potentials (spikes) were evoked using a series of 1-second long depolarizing current injections. Rheobase was defined as the minimal current required to evoke a spike. Neurons were tested with current injections from 1x rheobase to 4x rheobase using increments of 0.5x rheobase. Repetitive spiking neurons were defined as those producing ≥3 spikes in response to any stimulus intensity; transient spiking neurons consistently produced ≤2 spikes. Spike threshold was defined as voltage where dV/dt first exceeds 5 mV/ms (94). Spike height was measured from threshold to peak of the action potential. Only neurons with a resting membrane potential below -45 mV, spikes overshooting 0 mV and recordings with <20% change in series resistance were tested and analyzed. For dynamic clamp experiments, the pipette shank was painted with Sylgard (Dow) to reduce pipette capacitance. Virtual $Na_V1.7$ and $Na_V1.8$ conductances were introduced into the cells using CED software Signal v6. Currents were defined using the Hodgkin-Huxley equation, using the same parameter values as in our computational model (see below).

For voltage-clamp recordings, the bath solution was adjusted to reduce sodium currents to ensure proper clamping (85). Bath solution contained (in mM): 65 NaCl, 50 choline chloride, 5 KCl, 5 HEPES, 5 $MgCl_2$, 10 glucose, and 0.1 $CaCl_2$, plus 0.1 $CdCl_2$ to block calcium currents, and 20 TEA and 5 4-AP to block potassium currents; pH was adjusted to 7.4 with NaOH. Pipettes were filled with intracellular solution containing (in Mm): 140 CsCl, 10 HEPES, 2 $MgCl_2$, 1 EGTA, 3.8 Na-ATP, 0.4 Na-GTP; pH was adjusted to 7.3 with CsOH. The resulting pipette resistance was ~3 MΩ. A liquid junction potential correction of 4.8 mV was applied to all command voltages. Sodium currents

were recorded during 20 ms-long steps from -85 mV to voltages between -45 and +15 mV. Series resistance was compensated to >80%. Signals were amplified, low-pass filtered at 5 kHz, and digitized as described for current clamp recordings.

For all in vitro pharmacology, drugs were bath applied at a concentration chosen to selectively block the Na_V subtype of interest based on published EC50 values (see **Supplementary Table 3**)

Quantitative reverse transcription PCR (RT-qPCR)

Cultured DRG neurons <25 μm and expressing eYFP were identified as described above for patching. Coverslips were perfused with aCSF made with DEPC-treated ddH_2O, and identified neurons were collected using a glass pipette filled with intracellular solution also made from DEPC-treated ddH_2O (composition otherwise the same as described above for electrophysiology). Approximately 50 neurons were collected at DIV0 and at DIV4-7. Total mRNA was extracted with a PureLink RNA mini kit after digestion of genomic DNA with DNase I (Thermo Fisher Scientific) and the cDNA was synthesized with a SuperScript II first-strand synthesis kit (Thermo Fisher Scientific) according to instructions. RT-qPCR was performed with the cDNA primers of target genes (**Supplementary Table 3**), and the PowerUp SYBR® Green master mix (Thermo Fisher Scientific) in the QuantStudio-3 real-time PCR system. The primers were designed with IDT and spanned at least one exon longer than 1000 bp in order to exclude contamination from genomic DNA. Non-RT mRNA was also used as a negative control to exclude contamination from genomic DNA. All target genes were performed in triplicate for each sample and the experiments were repeated at least 3 times. $Na_V1.7$ and $Na_V1.8$ transcript levels were analyzed using the 2-ΔΔCT method and compared with the housekeeping gene HPRT.

Immunocytochemistry

Cultured DRG neurons were treated with 4% paraformaldehyde for 10 minutes, rinsed 3x with cold PBS, and permeabilized with 0.1% Triton X-100 in PBS. After another 3x rinse with PBS, neurons were treated with 10% normal goat serum for 30 min followed with rabbit primary $Na_V1.7$ antibody (1:200, ASC-008, Alomone) or $Na_V1.8$ antibody (1:200, ASC-028, Alomone) in PBS with 0.1% Tritween-20 and 1% BSA for 1 h. For some of the coverslips, primary antibodies were replaced with control peptides (ASC008AG1040 for $Na_V1.7$ and ASC016AG0640 for $Na_V1.8$) provided by Alomone as negative controls. Following 3x rinse in PBS, neurons were incubated in the dark with goat anti-rabbit secondary antibody Alexa Fluor-647 (1:500, Abcam) in PBS containing 1% BSA for 1 h, followed by DAPI staining for 10 min. All incubations were done at room temperature. Finally, coverslips were mounted on slides with mounting media (Abcam, ab128982), imaged with a spinning disk confocal microscope (Quorum Technologies) using the same acquisition setting across all imaging sessions, and analyzed with Volocity software (v6.5.1). Protein levels are measured using fluorescence intensity and expressed relative to each other (e.g. ratios in **Fig 6C**) or relative to fluorescence intensity for YFP in the same cells. Each condition was tested in a minimum of 3 animals.

Behavioral testing

Behavioral tests were performed on adult mice (male and female, 8-12 weeks). Mice were acclimated to the testing environment for at least 1 h the day prior to start of experiments. Behavioral testing (von Frey test and Hargreaves test) was then performed for 2-3 consecutive days for baseline and for another 3 days after CFA injection. Behavioral tests were performed at the same time in the morning, at room temperature (21°C) following a one-hour acclimation period. Animals were randomly assigned to experimental groups and the experimenter was blind to the drug condition.

CFA injection. CFA (Sigma, F5881) was thoroughly dissolved in saline (1:1) by vortexing the mixture. The resulting CFA solution (20 µl) was injected subdermally into the left hind paw under light isoflurane anaesthesia. The injection was performed shortly after the last baseline test, on Day 0.

PF-71 administration. Injectable PF-71 solution was prepared by first dissolving PF-71 in DMSO to make a 5% stock solution; dissolution was achieved by heating to 37°C and vortexing. On the day of injection, stock solution was dissolved in sunflower oil (5% v/v) by sonicating for 5 min. Freshly prepared final PF-71 solution was injected intraperitoneally (1 g/kg body weight). Behavioral testing was performed 2 hours after injection of PF-71 or vehicle.

Von Frey testing. Mechanical hyperalgesia was assessed with von Frey filaments (North Coast) using the SUDO method (95). The average of 3 trials in each animal was used for analysis.

Hargreaves testing. Thermal hyperalgesia was assessed with the Hargreaves apparatus (Ugo Basile, Italy). Radiant heat was applied to the plantar surface of the left hind paw. Interval between stimulus onset and paw withdrawal was defined as paw withdrawal latency (PWL). A 20 s cut-off prevented damage to the skin if the animal failed to withdraw. The average of 3 trials in each animal was used for analysis.

Statistical analysis

Results are expressed as mean ± SEM when data are normally distributed or otherwise as median and quartiles. Normality was tested using the Kolmogorov-Smirnov test. Analysis was performed with GraphPad Prism (v9) and SigmaPlot (v11). Normally distributed data were compared using t-tests or two-way ANOVAs followed by a Student Newman-Keuls post hoc test. Non-normally distributed data were compared using Mann-Whitney and Wilcoxon signed rank tests. Fisher exact and McNemar test were used for categorical data. Exact significance values and test results are reported throughout figure legends.

Computer model

Two separate, single compartmental models were built for DIV 0 and DIV4-7. They have the same, seven conductances: $g_{Nav1.3}$, $g_{Nav1.7}$, $g_{Nav1.8}$, g_{Kdr}, g_{M}, g_{AHP} and g_{Leak}. Channel equations and their gating parameters are provided in **Supplementary Table 4**. Conductance densities at baseline (**Supplementary Table 5**) were tuned to qualitatively reproduce the changes in Na_V channel expressions at DIV 0 and 4-7 indicated by the experiments; changes to other channels were minimized between the two models. The effects of ICA, PF-71 and PF-24 were simulated by adjusting $\bar{g}_{Nav1.3}$, $\bar{g}_{Nav1.7}$ and $\bar{g}_{Nav1.8}$, respectively, as reported in the figures. To re-introduce voltage noise that is otherwise missing from simulations, we included an Ornstein-Uhlenbeck process with μ_{noise} = 0 µA/cm^2, σ_{noise} = 0.05 µA/cm^2, and τ = 5 ms. All computer code is available at ModelDB (*http://modeldb.yale.edu/267560*; password: excitability). All simulations were conducted in MATLAB using the forward Euler integration method and a time step of 0.05–0.1 ms.

Contributions

YX, SR, SAP designed the research; YX, JY collected data, YX, JY, SR, SAP analyzed data; YX, SR, SAP wrote the manuscript.

Acknowledgements

This work was supported by a Restracomp fellowship to JY and by a Canadian Institutes of Health Research Foundation Grant (FDN167276) to SAP. We thank Rohini Kuner for providing $Na_V1.8$-Cre mice, Jason Jeong and Russell Smith for expert technical assistance with animal care and cell cultures, and Yongqian Wang for advice on qPCR data acquisition.

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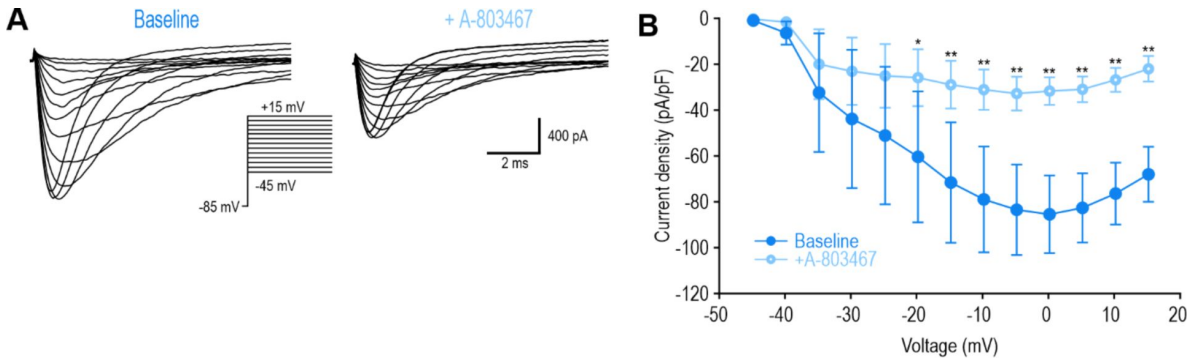

Figure 2 – figure supplement 1.

Inhibiting $Na_V1.8$ at DIV0 with A-803467 had the same effect as PF-24.

(A) Sample voltage clamp recording at DIV0 before and after A-803467 (1 μM). **(B)** Peak current was significantly reduced by A-803467 ($F_{1,84}$=9.935, p=0.016, two-way RM ANOVA, n=8). *, p<0.05; **, p<0.01; Student-Newman-Keuls post-hoc test in B.

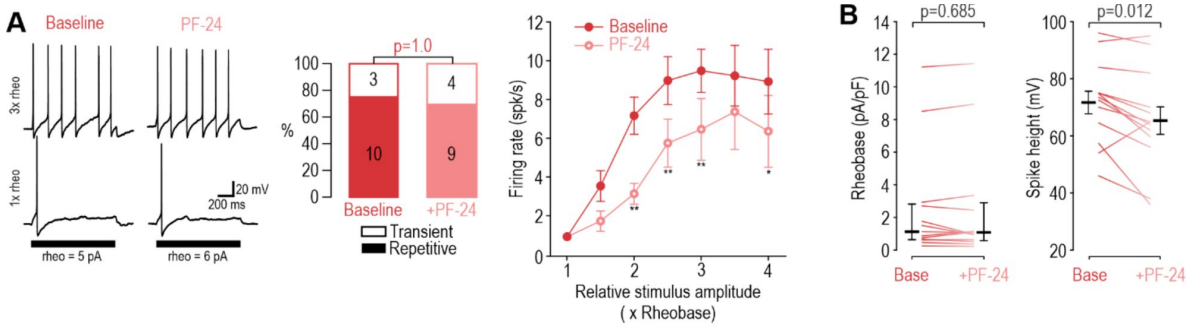

Figure 2 – figure supplement 2.

Inhibiting $Na_V1.8$ with PF-24 at DIV4-7 had negligible effects.

(A) Inhibiting $Na_V1.8$ with PF-24 (1 μM) did not affect spiking pattern (χ^2 =0.00, p=1.00, McNemar test) and modestly reduced firing rate ($F_{1,54}$=9.745, p= 0.012, two-way RM ANOVA, n=10) in DIV4-7 neurons. **(B)** PF-24 did not affect rheobase (Z_{12}=0.420, p=0.685, Wilcoxon Rank test) but did reduce spike height (T12=2.939, p=0.012, paired-t-test). *, p<0.05; **, p<0.01; Student-Newman-Keuls post-hoc tests in A.

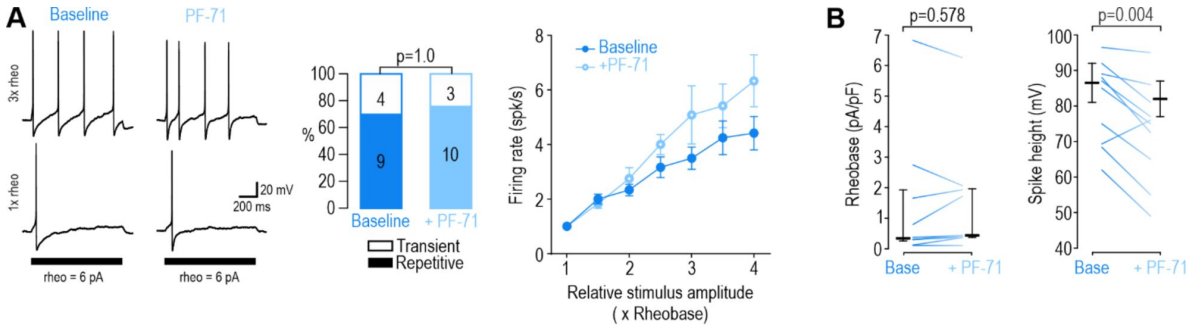

Figure 3 – figure supplement 1.

Inhibiting $Na_V1.7$ at DIV0 had negligible effects.

(A) Inhibiting $Na_V1.7$ with PF-71 (30 nM) did not alter spiking pattern (χ^2=0.00, p=1.00, McNemar test) or reduce firing rate ($F_{1,30}$=5.805, p=0.061, two-way RM ANOVA, n=6) in DIV0 neurons; in fact, firing rate was slightly increased. **(D-E)** PF-71 did not affect rheobase (Z_9=0.677, p=0.578, Wilcoxon rank test) but did reduce spike height (T_9=3.759, p=0.004, paired-t-test).

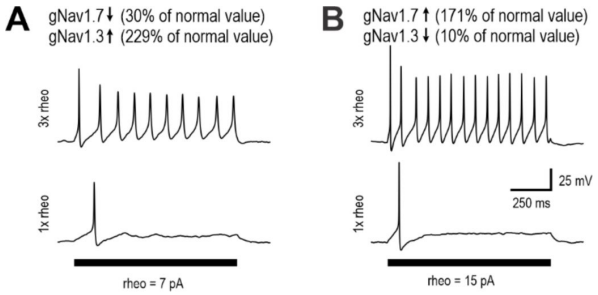

Figure 3 – figure supplement 2.

$Na_V1.7$ and $Na_V1.3$ currents can compensate for each other.

(A) In the DIV4-7 model, reducing $g_{Nav1.7}$ to 30% of its normal value (35→10 mS/cm^2) can be compensated for by increasing $g_{Nav1.3}$ to 229% of its normal value (0.35→0.8 mS/cm^2) to maintain repetitive spiking. **(B)** Conversely, reducing $g_{Nav1.3}$ to 10% of its normal value (0.35→0.035 mS/cm^2) can be compensated for by increasing $g_{Nav1.7}$ to 171% of its normal value (35→60 mS/cm^2) and maintain repetitive spiking.

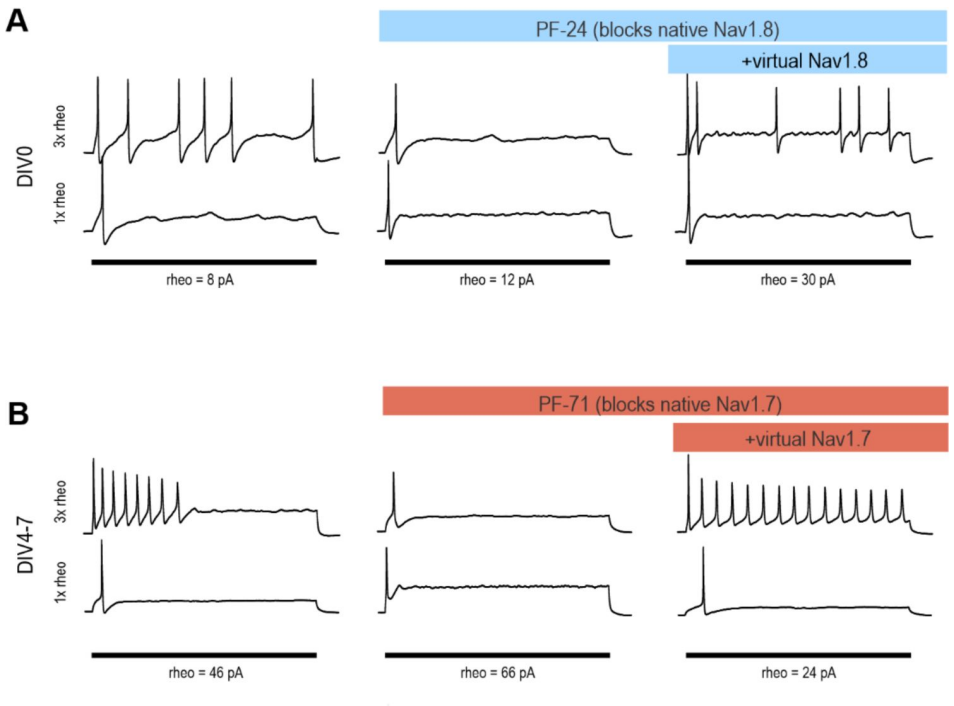

Figure 4 – figure supplement 1.

Virtual conductances restored repetitive spiking after pharmacological inhibition of the corresponding native conductance had converted the neuron to transient spiking.

(A) Sample response at DIV0 showing that a virtual $Na_V1.8$ conductance applied with dynamic clamp restored repetitive spiking after inhibiting native $Na_V1.8$ channels with PF-24. This restoration was repeated in 3 of 3 neurons tested. **(B)** Sample recording at DIV4-7 showing that a virtual $Na_V1.7$ conductance restored repetitive spiking after inhibiting native $Na_V1.7$ channels with PF-71. This restoration was repeated in 4 of 4 neurons tested.

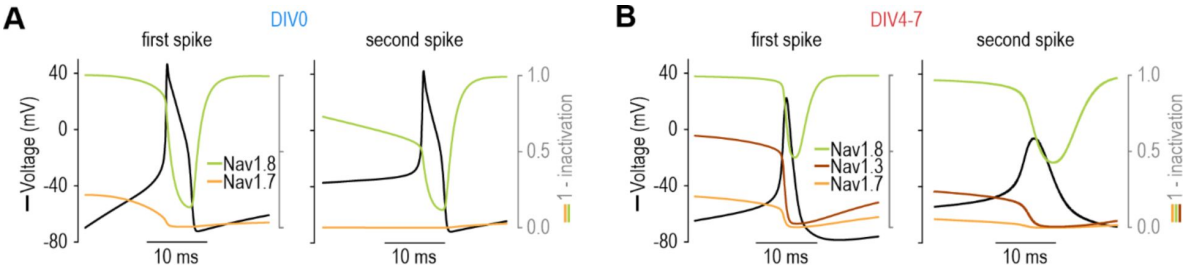

Figure 5 – figure supplement 1.

Channel inactivation affects Na_V subtype contribution on short timescale.

(A) In the DIV0 model, $Na_V1.7$ contributed to the first spike but its inactivation meant that all subsequent spikes relied exclusively on $Na_V1.8$. **(B)** In the DIV4-7 model, despite some inactivation of $Na_V1.3$ (maroon) and $Na_V1.7$ (orange), the remaining current was still large enough (because of the higher g_{max} of those two subtypes) to produce inward current sufficient to support repetitive spiking despite the low g_{max} of $Na_V1.8$ in the DIV4-7 model.

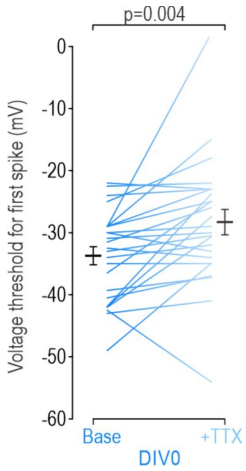

Figure 5 – figure supplement 2.

Effect of TTX on voltage threshold in DIV0 neurons.

Despite TTX having negligible effects in DIV0 neurons according to our initial analysis (see **Fig. 1**), simulation results in **Fig. 5A,B** predicted that the first spike was nonetheless initiated by $Na_V1.7$. By extension, this predicted that TTX should cause a depolarizing shift in voltage threshold for the first spike. Analysis of the experimental data confirmed this to be true, with threshold (mean±SEM) increasing from -33.7±1.4 mV at baseline to -28.3±1.4 mV after TTX (T_{24}=-3.19, p=0.004, paired t-test). Confirmation of this unexpected prediction helps further validate our model neuron.

	DIV0		DIV4-7		
	Baseline	↓$Na_V1.8$	Baseline	↓$Na_V1.7$	↓$Na_V1.3$
Rheobase (pA)	17	22	12	16	21
Spike height (mV)	72.8	46.9	57.1	21.4	52
RMP (mV)	-69.0	-69.7	-70.0	-69.8	-70.4

Supplementary Table 1.

Model data before and after channel “inhibition”

Reagent	Description	Source
TTX	Tetrodotoxin citrate	Alomone
PF-24	PF-01247324	Sigma
PF-71	PF-05089771	Alomone
ICA	ICA-121431	Tocris
Papain	Papain lyophilized power	Worthington
Collagenase	Collagenase type II	Worthington
Dispase II	Dispase type II	Sigma

Supplementary Table 2.

Reagents

Drug (concentration used)	EC50 values			References
	$Na_V1.3$	$Na_V1.7$	$Na_V1.8$	
TTX (100 nM)	**4 nM**	**2 nM**	46±10 μM	(21, 96)
ICA-121431 (1 μM)	**20 nM**	>10 μM	>10 μM	(53)
PF05089771 (30 nM)	11 μM	**11 nM**	>10 μM	(40)
PF-01247324 (1 μM)	>30 μM	>30 μM	**331 nM**	(50)
A-803467 (1 μM)	2.5 μM	6.7 μM	**8 nM**	(97)

Bolded entries indicate the Na_V subtype that is selectively blocked

Supplementary Table 3.

EC50 values

	HPRT	$Na_V1.7$	$Na_V1.8$
NCBI Reference Sequence	*NM_013556.2*	*NM_001290674.1*	*NM_001205321.1*
Length	144	114	148
Forward	TCCTCCTCAGACCGCTTT	TGATGGTCATGGTGATTGGG	AGCCATCAAAGTGTCCGTC
Reverse	TTTTCCAAATCCTCGGCATAATG	TGTTTGCGTCGGTGTCTTC	CTTTATCAGAGCCTCGAAGGTG

Supplementary Table 4.

Primers

Channel Model	Equations
$Na_V1.7$ (86)	$I_{NaV1.7} = \bar{g}_{NaV1.7} m^3 h (V - E_{Na})$ $\dot{m} = \alpha_m(1-m) - m\beta_m$ $\quad$ $\dot{h} = \alpha_h(1-h) - h\beta_h$ $\alpha_m = 10.22 - \frac{10.22}{1+\exp\left(\frac{V+7.19}{15.43}\right)}$ $\quad$ $\alpha_h = \frac{0.0744}{1+\exp\left(\frac{V+99.76}{11.07}\right)}$ $\beta_m = \frac{23.76}{1+\exp\left(\frac{V+70.37}{14.53}\right)}$ $\quad$ $\beta_h = 2.54 - \frac{2.54}{1+\exp\left(\frac{V+7.8}{10.68}\right)}$
$Na_V1.3$*	$I_{NaV1.3} = \bar{g}_{NaV1.3} m^3 h (V - E_{Na})$ $\dot{m} = \alpha_m(1-m) - m\beta_m$ $\quad$ $\dot{h} = \alpha_h(1-h) - h\beta_h$ $\alpha_m = 10.22 - \frac{10.22}{1+\exp\left(\frac{V+7.19+12}{15.43}\right)}$ $\quad$ $\alpha_h = \frac{0.0744}{1+\exp\left(\frac{V+99.76}{11.07}\right)}$ $\beta_m = \frac{23.76}{1+\exp\left(\frac{V+70.37+12}{14.53}\right)}$ $\quad$ $\beta_h = 2.54 - \frac{2.54}{1+\exp\left(\frac{V+7.8}{10.68}\right)}$
$Na_V1.8$ (87)	$I_{NaV1.8} = \bar{g}_{NaV1.8} m^3 h (V - E_{Na})$ $\dot{m} = \alpha_m(1-m) - m\beta_m$ $\quad$ $\dot{h} = \alpha_h(1-h) - h\beta_h$ $\alpha_m = 7.21 - \frac{7.21}{1+\exp\left(\frac{V-0.063}{7.86}\right)}$ $\quad$ $\alpha_h = 0.003 + \frac{1.63}{1+\exp\left(\frac{V+68.5}{10.01}\right)}$ $\beta_m = \frac{7.4}{1+\exp\left(\frac{V+53.06}{19.34}\right)}$ $\quad$ $\beta_h = 0.81 - \frac{0.81}{1+\exp\left(\frac{V-11.44}{13.12}\right)}$
K_M (88)	$I_{KM} = \bar{g}_{KM} n (V - E_K)$ $\dot{n} = \frac{n_\infty - n}{\tau_n}$ $n_\infty = \frac{1000}{1+e^{-\frac{V+35}{5}}}$ $\quad$ $\tau_n = \frac{1000}{3.3e^{V+\frac{35}{10}} + e^{-\frac{V+35}{10}}}$
K_{dr} (88)	$I_{KA} = \bar{g}_{KA} n^3 l (V - E_K)$ $\dot{n} = \alpha_m(1-m) - m\beta_m$ $\quad$ $\dot{l} = \alpha_h(1-h) - h\beta_h$ $\alpha_n = \frac{e^{(-5\times10^{-3}\cdot(V+32)\cdot9.648\times10^4)}}{2562.35}$ $\quad$ $\alpha_l = \frac{e^{(2\times10^{-3}\cdot(V+61)\cdot9.648\times10^4)}}{2562.35}$ $\beta_n = \frac{e^{(-2\times10^{-3}\cdot(V+32)\cdot9.648\times10^4)}}{2562.35}$ $\quad$ $\beta_l = \frac{e^{(-2\times10^{-3}\cdot(V+32)\cdot9.648\times10^4)}}{2562.35}$
AHP**	$I_{AHP} = \bar{g}_{AHP} z (V - E_K)$ $\quad$ $\dot{z} = \frac{1}{1+e^{5-V/4}} - \frac{z}{100}$

* Modified from $Na_V1.7$: a hyperpolarizing shift of 12mV in V1/2 of activation gate, m

** Modified from (49)

Note that the $Na_V1.7$ and $Na_V1.8$ equations above do not include the liquid junction potential correction of 4.2 mV (86) and 5.3 mV (87), respectively, that were applied in the neuron model.

Supplementary Table 5.

Model equations

	Conductance densities at baseline (mS/cm^2)						
	$\bar{g}_{\mathrm{Nav1.3}}$	$\bar{g}_{\mathrm{Nav1.7}}$	$\bar{g}_{\mathrm{Nav1.8}}$	$\bar{g}_{\mathrm{Kdr}}$	$\bar{g}_{\mathrm{M}}$	$\bar{g}_{\mathrm{AHP}}$	$\bar{g}_{\mathrm{Leak}}$
DIV 0	0	3	30	3	0.05	3.5	0.025
DIV 7	0.35	35	0.2	3.5	0.5	2.5	0.035

Supplementary Table 6.

Conductance densities at baseline for DIV 0 and 4-7 models

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Reviewer #1 (Public Review):

Summary:

In this work, Xie, Prescott and colleagues have reevaluated the role of Nav1.7 in nociceptive sensory neurons excitability. They find that nociceptors can make use of different sodium channel subtypes to reach equivalent excitability. The existence of this degeneracy is critical to understanding the neuronal physiology under normal and pathological conditions and could explain why Nav subtype-selective drugs have failed in clinical trials. More concretely,

nociceptor repetitive spiking relies on Nav1.8 at DIV0 (and probably under normal conditions in vivo), but on Nav1.7 and Nav1.3 at DIV4-7 (and after inflammation in vivo).

The conclusions of this paper are mostly well supported by data, and these findings should be of broad interest to scientists working on pain, drug development, neuronal excitability and ion channels.

Strengths:

The authors have employed elegant electrophysiology experiments (including specific pharmacology and dynamic clamp) and computational simulations to study the excitability of a subpopulation of DRGs that would very likely match with nociceptors (they take advantage of using transgenic mice to detect Nav1.8-expressing neurons). They make a strong point showing the degeneracy that occurs at the ion channel expression level in nociceptors, adding this new data to previous observations in other neuronal types. They also demonstrate that the different Nav subtypes functionally overlap and are able to interchange their "typical" roles in action potential generation. As Xie, Prescott and colleagues argue, the functional implications of the degenerate character of nociceptive sensory neurons excitability need to be seriously taken into account regarding drug development and clinical trials with Nav subtype-selective inhibitors.

In this revised version, the quality of the manuscript has been visibly improved. In my opinion, the questions and concerns raised by reviewers have been addressed clearly. After a detailed reading of this version and the comments to the reviewers, I have no additional comments or criticisms.

https://doi.org/10.7554/eLife.90960.2.sa2

Reviewer #2 (Public Review):

Summary:

The authors have noted in preliminary work that tetrodotoxin (TTX), which inhibits NaV1.7 and several other TTX-sensitive sodium channels, has differential effects on nociceptors, dramatically reducing their excitability under certain conditions but not under others. Partly because of this coincidental observation, the aim of the present work was to re-examine or characterize the role of NaV1.7 in nociceptor excitability and the effects on drug efficacy. The manuscript demonstrates that a NaV1.7-selective inhibitor produces analgesia only when nociceptor excitability is based on NaV1.7. More generally and comprehensively, the results show that nociceptors can achieve equivalent excitability through changes in differential NaV inactivation and NaV expression of different NaV subtypes (NaV 1.3/1.7 and 1.8). This can cause widespread changes in the role of a particular subtype over time. The degenerate nature of nociceptor excitability shows functional implications that make the assignment of pathological changes to a particular NaV subtype difficult or even impossible.

Thus, the analgesic efficacy of NaV1.7- or NaV1.8-selective agents depends essentially on which NaV subtype controls excitability at a given time point. These results explain, at least in part, the poor clinical outcomes with the use of subtype-selective NaV inhibitors and therefore have major implications for the future development of Nav-selective analgesics.

Strengths:

The results are clearly and impressively supported by the experiments and data shown. During the revision, the manuscript was consistently improved and the concerns of the first reviews were resolved. All methods are described in detail, and presumably, allow good reproducibility and were suitable to address the scientific question.

The results showing that nociceptors can achieve equivalent excitability through changes in differential NaV inactivation and expression of different NaV subtypes are of great importance in the fields of basic and clinical pain research and sodium channel physiology and pharmacology, but also for a broad readership and community. The degenerate nature of nociceptor excitability, which is clearly shown and well supported by data has large functional implications. The results are of great importance because they may explain, at least in part, the poor clinical outcomes with the use of subtype-selective NaV inhibitors and therefore have major implications for the future development of Nav-selective analgesics.

In summary, the authors achieved their overall aim to enlighten the role of the NaV1.7 in nociceptor excitability and the effects on drug efficacy. The data support the conclusions and clinical implications are highlighted as far as is currently justifiable due to the still limited experience in translation. This appears well-considered, not too speculative, and ultimately appropriate.

The main weaknesses of the first version were fixed during the revision:

(i) After revising the manuscript, the initial weakness that the computer model was described superficially has been fixed. Important information was added to the main text and additional information, including the full code and equations and values are deposited on ModelDB or are given in the Supplementary information (Suppl. Table 5 & 6).

(ii) The authors now comment that corresponding studies on protein levels or e.g. neuroinflammatory changes could support the characterization of the time course of membrane expression and cellular changes, but this should be addressed in future studies, as these analyses would also raise new questions, such as about membrane trafficking, post-translational modifications, etc. This is plausible and has now been appropriately addressed in the text.

(iii) During the initial review the authors were asked to discuss the promising role of NaV1.7 in the light of clinical results. In their response, the authors confidently state that they „wish to avoid speculating on which particular clinical results are better explained because our study was not designed for that." They, however, emphasize their take-home message, which is well supported "Instead, our take-home message (which is well supported; see Discussion on lines 309-321) is that NaV1.7-selective drugs may have a variable clinical effect because nociceptors' reliance on NaV1.7 is itself variable - much more than past studies would have readers believe. ... The challenge (as highlighted in the Abstract, lines 21-22) is that identifying the dominant Nav subtype to predict drug efficacy is difficult."

Against the background of this argumentation, it must be admitted that the decision not to present as yet unproven speculations is probably appropriate from a scientific point of view and that this ultimately proves the critical assessment of one's own data and the limitations of the study. This is undoubtedly acceptable and - in retrospect - probably the right way to go.

https://doi.org/10.7554/eLife.90960.2.sa1

Reviewer #3 (Public Review):

Summary:

In this study the authors used patch-clamp to characterize the implication of various voltage-gated Na+ channels in the firing properties of mouse nociceptive sensory neurons. They claim that depending on the culture conditions NaV1.3, NaV1.7, and NaV1.8 have distinct contributions to action potential firing and that similar firing patterns can result from distinct relative roles of these channels.

Strengths:

The paper addresses the important issue of understanding the lack of success of therapeutic strategies targeting NaV channels in the context of pain. Specifically, the authors test the hypothesis that different NaV channels contribute in a plastic manner to action potential firing, which may be the reason why it is difficult to target pain by inhibiting these channels.

Weaknesses:

(1) - The main claim of this paper is that "nociceptors can achieve equivalent excitability using different combinations of NaV1.3, NaV1.7, and NaV1.8". From this, they allude to the manifestation of "degeneracy", a concept implying that a biological process can occur via distinct sets of underlying components.
In my opinion, the analyses of the data is biased towards the author's interpretation.
- First, when comparing the excitability across neurons one should relate the response (in this case mean firing frequency) to the absolute size of the stimulus, not to the size of the stimulus normalized to the rheobase (see e.g., Figs. 1A). From this particular figure the authors conclude that the excitability is similar in the culture stages DIV0 and DIV4-7, but these data were not directly compared.
- Second, the authors reach their conclusion from the comparison of the (average) firing rate determined over 1 s current stimulation in distinct conditions. However, this is not the only parameter that determines how sensory neurons might convey information. For instance, the time dependence of the instantaneous frequency, the actual firing pattern, maybe also important.
- Third, the use of 1 s of current stimulation might not be sufficient to characterize the firing pattern if one wants to obtain conclusions that could translate to clinical settings (i.e., sustained pain).
- Fourth, out of principle, the gating properties of NaV1.7 and NaV1.8 channels are not identical, and therefore their contributions to excitability should not be the same. A neuron in which NaV1.7 is the main contributor is expected to have a damping firing pattern due to cumulative channel inactivation, whereas another depending mainly on NaV1.8 is expected to display more sustained firing. This is actually seen in the results of the modelling.

(2) - The quality of some recordings is dubious. The currents shown as TTX-sensitive in Fig. 1D look very strange (not like the ones at Baseline DIV4-7). These traces show abnormally fast inactivation and even transient deflections above zero current line. These are obvious artifacts of the subtraction procedure, probably due to unstable current amplitudes along the recording time. Similar odd-looking traces are shown in Fig. 3A.

(3) - I would like to point out that the main Significance Statement of the manuscript reads "The analgesic efficacy of subtype-selective drugs hinges on which subtype controls excitability". I would like to point out that, in addition of being extremely obvious for anyone knowing a bit about pain signaling, the authors did not test the analgesic efficacy of any drug in this study.

(4) - A critical issue in the manuscript is the unnecessary use of phrases that imply that biological entities have some sort of willpower, flirting with anthropomorphism and teleological language.
Sentences such as "Nociceptive sensory neurons convey pain signals to the CNS using action potentials" (see the Abstract) should be avoided. Neurons do not really "use" action potentials, they have no will to do so. Action potentials are not tools or means to be "used" by neurons. There are many other examples of misuse of the verb "use" in many other sentences. These were pointed out during the revision phase, but unfortunately the authors refused to correct them.

https://doi.org/10.7554/eLife.90960.2.sa0

Author Response

The following is the authors' response to the original reviews.

eLife assessment

This fundamental study provides an unprecedented understanding of the roles of different combinations of NaV channel isoforms in nociceptors' excitability, with relevance for the design of better strategies targeting NaV channels to treat pain. Although the experimental combination of electrophysiological, modeling, imaging, molecular biology, and behavioral data is convincing and supports the major claims of the work, some conclusions need to be strengthened by further evidence or discussion. The work may be of broad interest to scientists working on pain, drug development, neuronal excitability, and ion channels.

Reviewer #1 (Public Review):

Summary:

In this work, Xie, Prescott, and colleagues have reevaluated the role of Nav1.7 in nociceptive sensory neuron excitability. They find that nociceptors can make use of different sodium channel subtypes to reach equivalent excitability. The existence of this degeneracy is critical to understanding neuronal physiology under normal and pathological conditions and could explain why Nav subtype-selective drugs have failed in clinical trials. More concretely, nociceptor repetitive spiking relies on Nav1.8 at DIV0 (and probably under normal conditions in vivo), but on Nav1.7 and Nav1.3 at DIV4-7 (and after inflammation in vivo).

The conclusions of this paper are mostly well supported by data, and these findings should be of broad interest to scientists working on pain, drug development, neuronal excitability, and ion channels.

Strengths:

(1.1) The authors have employed elegant electrophysiology experiments (including specific pharmacology and dynamic clamp) and computational simulations to study the excitability of a subpopulation of DRGs that would very likely match with nociceptors (they take advantage of using transgenic mice to detect Nav1.8-expressing neurons). They make a strong point showing the degeneracy that occurs at the ion channel expression level in nociceptors, adding this new data to previous observations in other neuronal types. They also demonstrate that the different Nav subtypes functionally overlap and are able to interchange their "typical" roles in action potential generation. As Xie, Prescott, and colleagues argue, the functional implications of the degenerate character of nociceptive sensory neuron excitability need to be seriously taken into account regarding drug development and clinical trials with Nav subtype-selective inhibitors.

Weaknesses:

(1.2) The next comments are minor criticisms, as the major conclusions of the paper are well substantiated. Most of the results presented in the article have been obtained from experiments with DRG neuron cultures, and surely there is a greater degree of complexity and heterogeneity about the degeneracy of nociceptors excitability in the "in vivo" condition. Indeed, the authors show in Figures 7 and 8 data that support their

> *hypothesis and an increased Nav1.7's influence on nociceptor excitability after inflammation, but also a higher variability in the nociceptors spiking responses. On the other hand, DRG neurons targeted in this study (YFP (+) after crossing with Nav1.8-Cre mice) are >90% nociceptors, but not all nociceptors express Nav1.8 in vivo. As shown by Li et al., 2016 ("Somatosensory neuron types identified by high-coverage single-cell RNA-sequencing and functional heterogeneity"), there is a high heterogeneity of neuron subtypes within sensory neurons. Therefore, some caution should be taken when translating the results obtained with the DRG neuron cultures to the more complex "in vivo" panorama.*

We agree that most but not all Nav1.8+ DRG cells are nociceptors and that not all nociceptors express Nav1.8. We targeted small neurons that also express (or at some point expressed) Nav1.8, thus excluding larger neurons that express Nav1.8. This allowed us to hone in on a relatively homogeneous set of neurons, which is crucial when testing different neurons to compare between conditions (as opposed to testing longitudinally in the same neuron, which is not feasible). We expect all neurons are degenerate but likely on the basis of different ion channel combinations. Indeed, even within small Nav1.8+ neurons, other channels that we did not consider likely contribute to the degenerate regulation (as now better reflected in the revised Discussion).

That said, there are multiple sources of heterogeneity. We suspect that heterogeneity is more increased after inflammation than after axotomy because all DRG neurons experience axotomy when cultured whereas neurons experience inflammation differently in vivo depending on whether their axon innervates the inflamed area (now explained on lines 214-215). This is not so much about whether the insult occurs in vivo or in vitro, but about how homogeneously neurons are affected by the insult. Granted, neurons are indeed more likely to be heterogeneously affected in vivo since conditions are more complex. But our goal in testing PF-71 in behavioral tests (Fig. 8) was to show that changes observed in nociceptor excitability in Figure 7, despite heterogeneity, were predictive of changes in drug efficacy. In short, we establish Nav interchangeability by comparing neurons in culture (Figs 1-6), but we then show that similar Nav shifts can develop in vivo (Fig 7) with implications for drug efficacy (Fig 8). Such results should alert readers to the importance of degeneracy for drug efficacy (which is our main goal) even without a complete picture of nociceptor degeneracy or DRG neuron heterogeneity. Additions to the Discussion (lines 248-259, 304-308) are intended to highlight these considerations.

> *(1.3) Although the authors have focused their attention on Nav channels, it should be noted that degeneracy concerning other ion channels (such as potassium ion channels) could also impact the nociceptor excitability. The action potential AHP in Figure 1, panel A is very different comparing the DIV0 (blue) and DIV4-7 examples. Indeed, the conductance density values for the AHP current are higher at DIV0 than at DIV7 in the computational model (supplementary table 5). The role of other ion channels in order to obtain equivalent excitability should not be underestimated.*

We completely agree. We focused on Nav channels because of our initial observation with TTX and because of industry's efforts to develop Nav subtype-selective inhibitors, whose likelihood of success is affected by the changes we report. But other channels are presumably changing, especially given observed changes in the AHP shape (now mentioned on lines 304-308). Investigation should be expanded to include these other channels in future studies.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*

> *The authors have noted in preliminary work that tetrodotoxin (TTX), which inhibits NaV1.7 and several other TTX-sensitive sodium channels, has differential effects on nociceptors, dramatically reducing their excitability under certain conditions but not under others. Partly because of this coincidental observation, the aim of the present work was to re-examine or characterize the role of NaV1.7 in nociceptor excitability and its effects on drug efficacy. The manuscript demonstrates that a NaV1.7-selective inhibitor produces analgesia only when nociceptor excitability is based on NaV1.7. More generally and comprehensively, the results show that nociceptors can achieve equivalent excitability through changes in differential NaV inactivation and NaV expression of different NaV subtypes (NaV 1.3/1.7 and 1.8). This can cause widespread changes in the role of a particular subtype over time. The degenerate nature of nociceptor excitability shows functional implications that make the assignment of pathological changes to a particular NaV subtype difficult or even impossible.*
>
> *Thus, the analgesic efficacy of NaV1.7- or NaV1.8-selective agents depends essentially on which NaV subtype controls excitability at a given time point. These results explain, at least in part, the poor clinical outcomes with the use of subtype-selective NaV inhibitors and therefore have major implications for the future development of Nav-selective analgesics.*
>
> *Strengths:*
>
> *(2.1) The above results are clearly and impressively supported by the experiments and data shown. All methods are described in detail, presumably allow good reproducibility, and were suitable to address the corresponding question. The only exception is the description of the computer model, which should be described in more detail.*

We failed to report basic information such as the software, integration method and time step in the original text. This information is now provided on lines 476-477. Notably, the full code is available on ModelDB plus all equations including the values for all gating parameters are provided in Supplementary Table 5 and values for maximal conductance densities for DIV0 and DIV7 models are provided in Supplementary Table 6. Changes in conductance densities to simulate different pharmacological conditions are reported in the relevant figure legends (now shown in red). We did not include model details in the main text to avoid disrupting the flow of the presentation, but all the model details are reported in the Methods, tables and/or figure legends.

> *(2.2) The results showing that nociceptors can achieve equivalent excitability through changes in differential NaV inactivation and expression of different NaV subtypes are of great importance in the fields of basic and clinical pain research and sodium channel physiology and pharmacology, but also for a broad readership and community. The degenerate nature of nociceptor excitability, which is clearly shown and well supported by data has large functional implications. The results are of great importance because they may explain, at least in part, the poor clinical outcomes with the use of subtype-selective NaV inhibitors and therefore have major implications for the future development of Nav-selective analgesics.*
>
> *In summary, the authors achieved their overall aim to enlighten the role of NaV1.7 in nociceptor excitability and the effects on drug efficacy. The data support the conclusions, although the clinical implications could be highlighted in a more detailed manner.*
>
> *Weaknesses:*
>
> *As mentioned before, the results that nociceptors can achieve equivalent excitability through changes in differential NaV inactivation and NaV expression of different NaV*

> *subtypes are impressive. However, there is some "gap" between the DRG culture experiments and acutely dissociated DRGs from mice after CFA injection. In the extensive experiments with cultured DRG neurons, different time points after dissociation were compared. Although it would have been difficult for functional testing to examine additional time points (besides DIV0 and DIV47), at least mRNA and protein levels should have been determined at additional time points (DIV) to examine the time course or whether gene expression (mRNA) or membrane expression (protein) changes slowly and gradually or rapidly and more abruptly.*

Characterizing the time course of NaV expression changes is worthwhile but, insofar as such details are not necessary to establish that excitability is degenerate, it was not include in the current study. Furthermore, since mRNA levels do not parallel the functional changes in Nav1.7 (Figure 6A), we do not think it would be helpful to measure mRNA levels at intermediate time points. Measuring protein levels would be more informative, however, as now explained on lines 362-369, neurons were recorded at intermediate time points in initial experiments and showed a lot of variability. Methods that could track fluorescently-tagged NaV channels longitudinally (i.e. at different time points in the same cell) would be well suited for this sort of characterization, but will invariably lead to more questions about membrane trafficking, phosphorylation, etc. We agree that a thorough characterization would be interesting but we think it is best left for a future study.

> *It would also be interesting to clarify whether the changes that occur in culture (DIV0 vs. DIV47) are accompanied by (pro-)inflammatory changes in gene and protein expression, such as those known for nociceptors after CFA injection. This would better link the following data demonstrating that in acutely dissociated nociceptors after CFA injection, the inflammationinduced increase in NaV1.7 membrane expression enhances the effect of (or more neurons respond to) the NaV1.7 inhibitor PF-71, whereas fewer CFA neurons respond to the NaV1.8 inhibitor PF-24.*

These are some of the many good questions that emerge from our results. We are not particularly keen to investigate what happens over several days in culture, since this is not so clinically relevant, but it would be interesting to compare changes induced by nerve injury in vivo (which usually involves neuroinflammatory changes) and changes induced by inflammation. Many previous studies have touched on such issues but we are cautious about interpreting transcriptional changes, and of course all of these changes need to be considered in the context of cellular heterogeneity. It would be interesting to decipher if changes in NaV1.7 and NaV1.8 are directly linked so that an increase in one triggers a decrease in the other, and vice versa. But of course many other channels are also likely to change (as discussed above), and they too warrant attention, which makes the problem quite difficult. We look forward to tackling this in future work.

> *The results shown explain, at least in part, the poor clinical outcomes with the use of subtypeselective NaV inhibitors and therefore have important implications for the future development of Nav-selective analgesics. However, this point, which is also evident from the title of the manuscript, is discussed only superficially with respect to clinical outcomes. In particular, the promising role of NaV1.7, which plays a role in nociceptor hyperexcitability but not in "normal" neurons, should be discussed in light of clinical results and not just covered with a citation of a review. Which clinical results of NaV1.7-selective drugs can now be better explained and how?*

We wish to avoid speculating on which particular clinical results are better explained because our study was not designed for that. Instead, our take-home message (which is well supported; see Discussion on lines 309-321) is that NaV1.7-selective drugs may have a variable clinical effect because nociceptors' reliance on NaV1.7 is itself variable – much more than

past studies would have readers believe. At the end of the results (line 235), which is, we think, what prompted the reviewer's comment, we point to the Discussion. The corollary is that accounting for degeneracy could help account for variability in drug efficacy, which would of course be beneficial. The challenge (as highlighted in the Abstract, lines 21-22) is that identifying the dominant Nav subtype to predict drug efficacy is difficult. We certainly don't have all the answers, but we hope our results will point readers in a new direction to help answer such questions.

> *Another point directly related to the previous one, which should at least be discussed, is that all the data are from rodents, or in this case from mice, and this should explain the clinical data in humans. Even if "impediment to translation" is briefly mentioned in a slightly different context, one could (as mentioned above) discuss in more detail which human clinical data support the existence of "equivalent excitability through different sodium channels" also in humans.*

We are not aware of human data that speak directly to nociceptor degeneracy but degeneracy has been observed in diverse species; if anything, human neurons are probably even more degenerate based on progressive expansion of ion channel types, splice variants, etc. over evolution. Of course species differences extend beyond degeneracy and are always a concern for translation, because of a species difference in the drug target itself or because preclinical pain testing fails to capture the most clinically important aspects of pain (which we mention on line 35). Line 39 now reiterates that these explanations for translational difficulties are not mutually exclusive, but that degeneracy deserves greater consideration that is has hitherto received. Indeed, throughout our paper we imply that degeneracy may contribute to the clinical failure of Nav subtype-specific drugs, but those failures are certainly not evidence of degeneracy. In the Discussion (line 320-321), we now cite a recent review article on degeneracy in the context of epilepsy, and point out how parallels might help inform pain research. We wish we had a more direct answer to the reviewer's request; in the absence of this, we hope our results motivate readers to seek out these answers in future research.

> *Although speculative, it would be interesting for readers to know whether a treatment regimen based on "time since injury" with NaV1.7 and NaV1.8 inhibitors might offer benefits. Based on the data, could one hypothesize that NaV1.7 inhibitors are more likely to benefit (albeit in the short term) in patients with neuropathic pain with better patient selection (e.g., defined interval between injury and treatment)?*

We like that our data prompt this sort of prediction. However, this is potentially complicated since the injury may be subtle, which is to say that the exact timing may not be known. There are scenarios (e.g. postoperative pain) where the timing of the insult is known, but in other cases (e.g. diabetic neuropathy) the disease process is quite insidious, and different neurons might have progressed through different stages depending on how they were exposed to the insult. Our own experiments with CFA are a case in point. Notwithstanding the potential difficulties about gauging the time course, any way of predicting which Nav subtype is dominant could help more strategically choose which drug to use.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *In this study, the authors used patch-clamp to characterize the implication of various voltagegated Na+ channels in the firing properties of mouse nociceptive sensory neurons. They report that depending on the culture conditions NaV1.3, NaV1.7, and NaV1.8 have distinct contributions to action potential firing and that similar firing patterns can result from distinct relative roles of these channels. The findings may be relevant for the design of better strategies targeting NaV channels to treat pain.*

> *Strengths:*
>
> *The paper addresses the important issue of understanding, from an interesting perspective, the lack of success of therapeutic strategies targeting NaV channels in the context of pain. Specifically, the authors test the hypothesis that different NaV channels contribute in a plastic manner to action potential firing, which may be the reason why it is difficult to target pain by inhibiting these channels. The experiments seem to have been properly performed and most conclusions are justified. The paper is concisely written and easy to follow.*
>
> *Weaknesses:*
>
> *(1) The most critical issue I find in the manuscript is the claim that different combinations of NaV channels result in equivalent excitability. For example, in the Abstract it is stated that: "...we show that nociceptors can achieve equivalent excitability using different combinations of NaV1.3, NaV1.7, and NaV1.8". The gating properties of these channels are not identical, and therefore their contributions to excitability should not be the same. I think that the culprit of this issue is that the authors reach their conclusion from the comparison of the (average) firing rate determined over 1 s current stimulation in distinct conditions. However, this is not the only parameter that determines how sensory neurons convey information. For instance, the time dependence of the instantaneous frequency, the actual firing pattern, may be important too. Moreover, the use of 1 s of current stimulation might not be sufficient to characterize the firing pattern if one wants to obtain conclusions that could translate to clinical settings (i.e., sustained pain). A neuron in which NaV1.7 is the main contributor is expected to have a damping firing pattern due to cumulative channel inactivation, whereas another depending mainly on NaV1.8 is expected to display more sustained firing. This is actually seen in the results of the modelling.*

This concern seems to boil down to how equivalent is equivalent? The spike shape or the full inputoutput curve for a DIV0 neuron (Nav1.8-dominant) is never equivalent to what's seen in a DIV47 neuron (Nav1.7-dominant), but nor are any two DIV0 neurons strictly equivalent, and likewise for any two DIV4-7 neurons. Our point is that DIV0 and DIV4-7 neurons are a far more similar (less discriminable) in their excitability than expected from the qualitative difference in their TTX sensitivity (and from repeated claims in the literature that Nav1.7 is necessary for spike generation in nociceptors). Nav isoforms need not be identical to operate similarly; for instance, Nav1.8 tends to activate at "suprathreshold" voltages, but this depends on the value of threshold; if threshold increases, Nav1.8 can activate at subthreshold voltages (see Fig 5). We have modified lines 155- 175 to help clarify this.

We completely agree that firing rate is not the only way to convey sensory information, and of course injecting current directly into the cell body via a patch pipette is not a natural stimulus. These are all factors to keep in mind when interpreting our data. Nonetheless, our data show that excitability is similar between DIV0 and DIV 4-7, so much so that data from any one neuron (without pharmacological tests or capacitance measurements) would likely not reveal if that cell is DIV0 or DIV4-7; this "indiscriminability" qualifies as "equivalent" for our purposes, and is consistent with phrasing used by other authors studying degeneracy. Notably, not every DIV4-7 neuron exhibits spike height attenuation (see Fig. 1A), likely because of concomitant changes in the AHP that were not captured in our computer model or directly tested in our experiments. This highlights that other channel changes may also contribute to degeneracy and the maintenance of repetitive spiking.

> *(2) In Fig. 1, is 100 nM TTX sufficient to inhibit all TTX-sensitive NaV currents? More common in literature values to fully inhibit these currents are between 300 to 500 nM. The currents shown as TTX-sensitive in Fig. 1D look very strange (not like the ones at*

> *Baseline DIV4-7). It seems that 100 nM TTX was not enough, leading to an underestimation of the amplitude of the TTXsensitive currents.*

As now summarized in Supplementary Table 3 (which is newly added), 100 nM TTX is >20x the EC50 for Nav1.3 and Nav1.7 (but is still far below the EC50 for Nav1.8). Based on this, TTXsensitive channels are definitely blocked in our TTX experiments.

> *(3) Page 8, the authors conclude that "Inflammation caused nociceptors to become much more variable in their reliance of specific NaV subtypes". However, how did the authors ensure that all neurons tested were affected by the CFA model? It could be that the heterogeneity in neuron properties results from distinct levels of effects of CFA.*

We agree with the reviewer. We also believe that variable exposure to CFA is the most likely explanation for the heightened variability in TTX-sensitivity reported in Figure 7 (now more clearly explained on lines 214-215). One could try co-injecting a retrograde dye with the CFA to label cells innervating the injection site, but differential spread of the CFA and dye are liable to preclude any good concordance. Alternatively, a pain model involving more widespread (systemic) inflammation might cause a more homogeneous effect. But, our main goal with CFA injections was to show that a Nav1.8®Nav1.7 switch can occur in vivo (and is therefore not unique to culturing), and that demonstration is true even if some neurons do not switch. Subsequent testing in Figure 8 shows that enough neurons switch to have a meaningful effect in terms of the behavioral pharmacology. So, notwithstanding tangential concerns, we think our CFA experiments succeeded in showing that Nav channels can switch in vivo and that this impacts drug efficacy.

> ***Recommendations for the authors:***
>
> *All reviewers agreed that these results are solid and interesting. However, the reviewers also raised several concerns that should be addressed by the authors to improve the strength of the evidence presented. Revisions considered to be essential include:*
>
> *(1) Discuss how degeneracy concerning other ion channels (such as potassium ion channels) could also impact nociceptor excitability (reviewer #1). Additionally, the translation of results from DRG neuron cultures to "in vivo" nociceptors should be better discussed.*

We have added a new paragraph to the Discussion (line 248-259) to remind readers that despite our focus on Nav channels, other ion channels likely also change (and that these changes involve diverse regulatory mechanisms that require further investigation). Likewise, despite our focus on the changes caused by culturing neurons, we remind readers that subtler, more clinically relevant in vivo perturbations can likewise cause a multitude of changes. We end that paragraph by emphasizing that although accounting for all the contributing components is required to fully understand a degenerate system, meaningful progress can be made by studying a subset of the components. We want to emphasize this because there is some middle ground between focusing on one component at a time (which is the norm) vs. trying to account for everything (which is an infeasible ideal). Additional text on lines 304-308 also addresses related points.

> *(2) Discuss how different combinations of NaV channels result in equivalent excitability, in the context of the experimental conditions used (see main comment by reviewer #3). It should also be discussed in more detail which human clinical data support the existence of "equivalent excitability through different sodium channels" also in humans (reviewer #2).*

Regarding the first part of this comment, reviewer 3 wrote in the public review that “The gating properties of these channels are not identical, and therefore their contributions to excitability should not be the same.” Differences in gating properties are commonly used to argue that different Nav subtypes mediate different phases of the spike, for example, that Nav1.7 initiates the spike whereas Nav1.8 mediates subsequent depolarization because Nav1.7 and Nav1.8 activate at perithreshold and suprathrehold voltages, respectively (see lines 134-135, now shown in red). But such comparison is overly simplistic insofar as it neglects the context in which ion channels operate. For instance, if Nav1.7 is not expressed or fully inactivates, voltage threshold will be less negative, enabling Nav1.8 to contribute to spike initiation; in other words, previously “suprathreshold” voltages become “perithreshold”. Figure 5 is dedicated to explaining this context-sensitivity; specifically, we demonstrate with simulations how Nav1.8 takes over responsibility for initiating a spike when Na1.7 is absent or inactivated. Text on lines 155- 184 has been edited to help clarify this. Regarding the second part of this comment, we are not aware of any direct evidence from human sensory neurons that different sodium channels produce equivalent excitability, but that is certainly what we expect. We suggest that failure of Nav subtype-specific drugs is, at least in part, because of degeneracy, but such failures do not demonstrate degeneracy unless other contributing factors can be excluded (which they can’t). Recognizing degeneracy is difficult, and so variability that might be explained by degeneracy will go unexplained or attributed to other factors unless, by design or serendipity, experiments quantify the effects of degeneracy (as we have attempted to do here). We now cite a recent review article on degeneracy and epilepsy (line 320), which addresses relevant themes that might help inform pain research; for instance, most existing antiseizure medications act on multiple targets whereas more recently developed single-target drugs have proven largely ineffective. This is similar to but better documented than for analgesics. With this in mind, we revised the text to emphasize the circumstantial nature of existing evidence and the need to test more directly for degeneracy (lines 320-323).

> *(3) Extend the discussion about the poor clinical outcomes with the use of subtype-selective NaV inhibitors. In particular, the promising role of NaV1.7, which plays a role in nociceptor hyperexcitability but not in "normal" neurons, should be discussed in light of clinical results and not just covered with a citation of a review. Which clinical results of NaV1.7-selective drugs can now be better explained and how? (reviewer #2)*

As discussed above, we are cautious avoid speculating on which clinical results are attributable to degeneracy. Instead, our take-home message (see Discussion, lines 309-323) is that NaV1.7selective drugs may have a variable clinical effect because nociceptors’ reliance on NaV1.7 is itself variable – much more than past studies would have readers believe. The corollary is that accounting for degeneracy could help account for variability in drug efficacy, which would of course be beneficial. The challenge (as highlighted in the Abstract, lines 21-22) is that identifying the dominant Nav subtype to predict drug efficacy is not trivial. Interpreting clinical data is also complicated by the fact that we are either dealing with genetic mutations (with unclear compensatory changes) or pharmacological results (where NaV1.7-selective drugs have a multitude of problems that might contribute to their lack of efficacy, separate from effects of degeneracy). We have striven to contextualize our results (e.g. last paragraph of results, lines 222-235). We think this is the most we can reasonably say based on the limitations of existing clinical data.

> *(4) Provide a clearer and more detailed description of the computational model (reviewers #2 and #3).*

We added important details on line 476-477 but, in our honest opinion, we think our computational model is thoroughly explained. The issue seems to boil down to whether

details are included in the Results vs. being left for the Methods, tables and figure legends. We prefer the latter.

> *(5) Better clarify the effects of the CFA model, to provide further evidence relating inflammation with nociceptors variability (reviewers #2 and #3)*

As explained in response to a specific point by reviewer #3, we believe that variable exposure to CFA explains the heightened variability in TTX-sensitivity reported in Figure 7 (now explained on lines 214-215). One could try co-injecting a retrograde dye with the CFA to label cells innervating the injection site, but differential spread of the inflammation and dye are liable to preclude any good concordance. Alternatively, a pain model involving more widespread (systemic) inflammation might cause a more homogeneous effect. But, our main goal with CFA injections was to show that a Nav1.8®Nav1.7 switch can occur in vivo (and is therefore not unique to culturing); that demonstration holds true even if some neurons do not switch. Subsequent testing (Fig 8) shows that enough neurons switch to drug efficacy assessed behaviorally. This is emphasized with new text on lines 225-227. Overall, we think our CFA experiments succeed in showing that Nav channels can switch in vivo and, despite variability, that this occurs in enough neurons to impact drug efficacy.

> *(6) Revise the text according to all recommendations raised by the reviewers and listed in the individual reviews.*

Detailed responses are provided below for all feedback and changes to the text were made whenever necessary, as identified in our responses.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Minor points/recommendations:*
>
> *Protein synthesis inhibition by cercosporamide could be the direct cause of a smaller-thanexpected increase in Nav1.7 levels at DIV5. But for Nav1.8, there is a mitigation in the increased levels at DIV5, that only could be explained by several indirect mechanisms, including membrane trafficking and posttranslational modifications (phosphorylation, SUMOylation, etc.) on Nav1.8 or protein regulators of Nav1.8 channels. The authors suggest that "translational regulation is crucial", but also insinuate that other processes (membrane trafficking, etc.) could contribute to the observed outcome. It is difficult to assess the relative importance of these different explanations without knowing the exact mechanisms that are acting here.*

We agree. We relied on electrophysiology (and pharmacology) to measure functional changes, but we wanted to verify those data with another method. We expected mRNA levels to parallel the functional changes but, when that did not pan out, we proceeded to look at protein levels. Perhaps we should have stopped there, but by blocking protein translation, we show that there is not enough Nav1.7 protein already available that can be trafficked to the membrane. That does not explain why Nav1.8 levels drop. Our immunohistochemistry could not tease apart membrane expression from overall expression, which limits interpretation. We have enhanced the text to discuss this (lines 200-204), but further experiments are needed. Though admittedly incomplete, our initial finding help set the stage for future experiments on this matter.

> *Page 15, typo: "contamination from genomic RNA" -> "contamination from genomic DNA" (appears twice).*

This has been corrected on lines 420 and 421.

> *Page 17: I could not find the computer code at ModelDB (http://modeldb.yale.edu/267560). It seems to be an old web link. It should be available at some web repository.*

We confirmed that the link works. Entry is password-protected (password = excitability; see line 476). Password protection will be removed once the paper is officially published.

> *Page 19, reference 36, typo: "Inhibitio of" -> "Inhibition of".*

This has been corrected (line 557).

> *Page 33, typo: "are significantly larger than differences at DIV1" -> "are significantly larger than differences at DIV0".*

This has been corrected (line 796).

> *Page 35, figure 6 legend. The number of experiments (n) is not indicated for panel C data.*

N = 3 is now reported (line 828).

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *p. 3/4 and Data of Fig. 6: It should be commented on why days 1-3 were not investigated. An investigation of the time course (by higher frequency testing) would certainly have an added value because it would be possible to deduce whether the changes develop slowly and gradually, or whether the excitability induced by different NaVs changes suddenly. At least mRNA and protein levels should be determined at additional time points to examine the time course or whether gene expression (mRNA) or membrane expression (protein) changes slowly and gradually or rapidly and more abruptly. It would also be interesting to clarify whether the changes that occur in culture (DIV0 vs. DIV4-7) are accompanied by (pro-)inflammatory changes in gene and protein expression, such as those known for nociceptors after CFA injection. Or is the latter question clear in the literature?*

We now explain (lines 362-369) that intermediate time points (DIV1-3) were tested in initial current clamp recordings. Those data showed that TTX-sensitivity stabilized by DIV4 and differed from the TTX-insensitivity observed at DIV0. TTX-sensitivity was mixed at DIV1-3 and crosscell variability complicated interpretation. Subsequent experiments were prioritized to clarify why NaV1.7 is not always critical for nociceptor excitability, contrary to past studies. Our efforts to measure mRNA and protein levels were primarily to validate our electrophysiological findings; we are also interested in deciphering the underlying regulatory processes but this is an entire study on its own. Unfortunately, the existing literature does not help or point to an explanation for the Nav1.7/1.8 shift we observed.

Our evidence that mRNA levels do not parallel functional changes argues against pursuing transcriptional changes in Nav1.7, though transcriptional changes in other factors might be important. Interpretation of immuno quantification would be complicated by the high variability we observed with the physiology at intermediate time points and, furthermore, we cannot resolve surface expression from overall expression based on available antibodies. Methods conducive to longitudinal measurements would be more appropriate (as now mentioned on line 367-369). In short, a lot more work is required to understand the mechanisms involved in the switch, but we think the existing demonstration suffices to show that NaV1.7 and NaV1.8 protein levels vary, with crucial implications for which Nav subtype controls nociceptor excitability, and important implications for drug efficacy. Explaining why

and how quickly those protein levels change will be no small feat is best left for a future study.

> *p. 4 and following: In order to enable the interpretation of the used concentration of PF-24, PF71, and ICA, the respective IC50 should be indicated.*

A table (now Supplementary Table 3; line 861) has been added to report EC50 values for all drugs for blocking NaV1.7, NaV1.8 and NaV1.3. The concentrations we used are included on that table for easy comparison.

> *p. 5, end of the middle paragraph: Here it should be briefly explained -for less familiar readers- why NaV1.1 cannot be causative (ICA inhibits NaV1.1 and 1.3).*

We now explain (lines 117-120) that NaV1.1 is expressed almost exclusively in medium-diameter (A-delta) neurons whereas NaV1.3 is known to be upregulated in small-diameter neurons, and so the effect we observe in small neurons is most likely via blockade NaV1.3.

> *p. 6, lines 4/5: At least once it should read computer model instead of model.*

“Computer” has been added the first time we refer to DIV0 or DIV4-7 computer models (lines 138-139)

> *p. 6: the difference between Fig. 4B and Fig. 4 - Figure suppl. 1 should be mentioned briefly.*

We now explain (lines 150-154) that Fig. 4B involves replacing a native channel with a different virtual channel (to demonstrate their interchangeability) whereas and Fig. 4 - Figure supplement 1 involves replacing a native channel with the equivalent virtual channel (as a positive control).

p. 6/7: the text and the conclusions regarding Figure 5 are difficult to follow. Somewhat more detailed explanations of why which data demonstrate or prove something would be helpful.

The text describing Figure 5 (lines 155-175) has been revised to provide more detail.

> *p. 7, last sentence of the first paragraph: How is this supported by the data? Or should this sentence be better moved to the discussion?*

This sentence (now lines 182-184) is designed as a transition. The first half – “a subtype’s contribution shifts rapidly (because of channel inactivation)” – summarizes the immediately preceding data (Figure 5). The second half – “or slowly (because of [changes in conductance density])” – introduces the next section. The text show in square brackets has been revised. We hope this will be clearer based on revisions to the associated text.

> *p. 7, second paragraph, line 3: Please delete one "at both".*

Corrected

> *p. 7, second paragraph: Please explain why different time points (DIV4-7, DIV5, or DIV7) were used or studied.*

Initial electrophysiological experiments determined that TTX sensitivity stabilized by DIV 4 (see response to opening point) and we did not maintain neurons longer than 7 days, and so neurons recorded between DIV4 and 7 were pooled. If non-electrophysiological tests were conducted on a specific day within that range, we report the specific day, but any day within

the DIV4-7 range is expected to give comparable results. This is now explained on lines 365-367.

> *p. 8: the text regarding Fig. 7 should also include the important data (e.g. percentage of neurons showing repetitive spinking) mentioned in the legend.*

This text (lines 216-220) has been revised to include the proportion of neurons converted by PF71 and PF-24 and the associated statistical results.

> *Fig. 1: third panel (TTX-sensitive current...) of D & Fig. 2 subpanel of A (Nav1.8 current...). These panels should be explained or mentioned in the text and/or legends.*

We now explain in the figure legends (lines 708-710; 714-715; 736-738) how those currents are found through subtraction.

> *Fig. 2 - figure supplement 2. One might consider taking Panel A to Fig. 2 so that the comparison to DIV0 is apparent without switching to Suppl. Figs.*

We left this unchanged so that Figures 2 and 3 are equivalently organized, with negative control data left to the supplemental figures. Elife formatting makes it easy to reach the supplementary figure from the main figure, so we hope this won't be an impediment to readers.

> *Fig. 6 C, middle graph (graph of Nav1.7): Please re-check, whether DIV5 none vs. 24 h and none vs. 120 h are really significantly different with such a low p-value.*

We re-checked the statistics and the difference pointed out by the reviewer is significant at p=0.007. We mistakenly reported p<0.001 for all comparisons, and so this p value has been corrected; all the other p values are indeed <0.001. Notably, the data are summarized as median ± quartile because of their non-Gaussian distribution; this is now explained on line 827 (as a reminder to the statement on lines 461-462). Quartiles are more comparable to SD than to SEM (in that quartiles and SD represent the distribution rather than confidence in estimating the mean, like SEM), and so medians can differ very significantly even if quartiles overlap, as in this case.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *(1) A critical issue in the manuscript is the use of teleological language. It is likely that this is not the intention, but careful revision of the language should be done to avoid the use of expressions that confer purpose to a biological process. Please, find below a list of statements that I consider require correction.*
>
> - *In the Abstract, the first sentence: "Nociceptive sensory neurons convey pain signals to the CNS using action potentials". Neurons do not really "use" action potentials, they have no will or purpose to do so. Action potentials are not tools or means to be "used" by neurons. Other examples of misuse of the verb "use" are found in several other sentences:*
>
> *"...nociceptors can achieve equivalent excitability using different combinations of NaV1.3, NaV1.7, and NaV1.8"*
>
> *"Flexible use of different NaV subtypes - an example of degeneracy - compromises..."*
>
> *"Nociceptors can achieve equivalent excitability using different sodium channel subtypes"*
> *"...degeneracy - the ability of a biological system to achieve equivalent function using*

> *different components..."*
>
> *"...nociceptors can achieve equivalent excitability using different sodium channel subtypes..."*
>
> *"Our results show that nociceptors can achieve similar excitability using different NaV channels" "...the spinal dorsal horn circuit can achieve similar output using different synaptic weight combinations..."*
>
> *"Contrary to the view that certain ion channels are uniquely responsible for certain aspects of neuronal function, neurons use diverse ion channel combinations to achieve similar function" "In summary, our results show that nociceptors can achieve equivalent excitability using different NaV subtypes"*

"Use" can mean to put into action (without necessarily implying intention). Based on definitions of the word in various dictionaries, we feel we are well within the realm of normal usage of this term. In trying to achieve a clear and succinct writing style, we have stuck with our original word choice.

> - *At the end of page 5 and in the legend of Fig. 7, the word "encourage" is not properly used in the sentence "The ability of NaV1.3, NaV1.7 and NaV1.8 to each encourage repetitive spiking is seemingly inconsistent with the common view...". Encouraging is really an action of humans or animals on other humans or animals.*

Like for "use", we verified our usage in various dictionaries and we do not think that most readers will be confused or disturbed by our word choice. We use "encourage" to explain that increasing NaV1.3, NaV1.7 or NaV1.8 can increase the likelihood of repetitive spiking; we avoided "cause" because the probability of repetitive spiking is not raised to 100%, since other factors must always be considered.

> - *In the Abstract and other places in the manuscript, the word "responsibility" seems to be wrongly employed. It is true that one can say, for instance, on page 4 last paragraph "we sought to identify the NaV subtype responsible for repetitive spiking at each time point". However, to confer channels with the human quality of having "responsibility" for something does not seem appropriate. See also page 8 last paragraph, the first paragraph of the Discussion, and the three paragraphs of page 11.*

Again, we must respectfully disagree with the reviewer. We appreciate that this reviewer does not like our writing style but we do not believe that our style violates English norms.

> *(2) In the first sentence of the Abstract, nociceptive sensory neurons do not convey "pain signals". Pain is a sensation that is generated in the brain.*

"Pain" is used as an adjective for "signal" and is used to help identify the type of signal. Nonetheless, since the word count allowed for it, we now refer to "pain-related signals" (line 10).

> *(3) I do not see the point of plotting the firing rate as a function of relative stimulus amplitude (normalized to the rheobase, e.g., Fig. 1A bottom panels, Fig. 2B, bottom-right, Fig. 2 Supp2A right, Fig. 3 B bottom panels, etc) instead of as a function of the actual stimulus amplitude. I have the impression that this maneuver hides information. This is*

> *equivalent to plotting the current amplitudes as a function of the voltage normalized by the voltage threshold for current activation, which is obviously not done.*

This is how the experiments were performed, so it would be impossible to perform the statistical analysis using the absolute amplitudes post-hoc; specifically, stimulus intensities were tested at increments defined relative to rheobase rather than in absolute terms. There are pros and cons to each approach, and both approaches are commonly used. Notably, we report the value of rheobase on the figures so that readers can, with minimal arithmetic, convert to absolute stimulus intensities. No information is hidden by our approach.

> *(4) On page 4 it is stated that "We show later that similar changes develop in vivo following inflammation with consequences for drug efficacy assessed behaviourally (see Fig. 8), meaning the NaV channel reconfiguration described above is not a trivial epiphenomenon of culturing". However, what happens in culture may have nothing in common with what happens in vivo during inflammation. Thus, the latter data may not serve to answer whether the culture conditions induce artifacts or not. I suggest tuning down this statement by changing "meaning" to "suggesting".*

On line 97, we now write “suggesting”.

> *(5) Page 5, first paragraph, I miss a clear description of the mathematical models. Having to skip to the Methods section to look for the details of the models as the artifices introduced to simulate different conditions is rather inconvenient.*

So as not to disrupt the flow of the presentation with methodological details, we only provide a short description of the model in the Results. We have slightly expanded this to point out that the conductance-based model is also single-compartment (line 111). We provide a very thorough description of our model in the Methods, especially considering all the details provided in Supplementary Tables 1, 5 and 6. We also report conductance densities and % changes in figure legends (lines 722, 747-748; now shown in red). This is also true for Figure 3-figure supplement 2 (lines 756-759). We tried very hard to find a good balance that we hope most readers will appreciate.

> *(6) Page 6, second paragraph, simulations do not serve to "measure" currents.*

The sentence been revised to indicate that simulations were used to “infer” currents during different phases of the spike (line 155).

> *(7) Page 7, regarding the tile of the subsection "Control of changes in NaV subtype expression between DIV0 and DIV4-7", the authors measured the levels of expression, but not really the mechanisms "controlling" them. I suggest writing "changes in NaV subtype expression between DIV0 and DIV4-7"*

We have removed “control of” from the section title (line 185)

> *(8) What was the reason for adding a noise contribution in the model?*

We now explain that noise was added to reintroduce the voltage noise that is otherwise missing from simulations (line 474). For instance, in the absence of noise, membrane potential can approach voltage threshold very slowly without triggering a spike, which does not happen under realistically noisy conditions. Of course membrane potential fluctuates noisily because of stochastic channel opening and a multitude of other reasons. This is not a major issue for this study, and so we think our short explanation should suffice.

> *(9) Please, define the concept of degeneracy upon first mention.*

Degeneracy is now succinctly defined in the abstract (line 20).