# EQUIVALENT EXCITABILITY THROUGH DIFFERENT SODIUM CHANNELS AND IMPLICATIONS FOR THE ANALGESIC EFFICACY OF SELECTIVE DRUGS

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**Short Title:** Implications of nociceptor degeneracy

## ABSTRACT

Nociceptive sensory neurons convey pain 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 – an example of degeneracy – 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.

## 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), which has triggered 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).

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 ($\chi^2$=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), meaning the $Na_V$ channel reconfiguration described above is not a trivial epiphenomenon of culturing.

***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 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). 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 $Na_V1.1/1.3$ are both necessary for repetitive spiking at DIV4-7. Inhibiting $Na_V1.7$ increased rheobase, unlike inhibiting $Na_V1.1/1.3$, 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 55). 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 model could be replaced with $Na_V1.7$, and whether the $Na_V1.7$ current necessary for spiking in our DIV4-7 model could be replaced 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.

With the model neurons thus validated, we used simulations to measure $Na_V1.7$ and $Na_V1.8$ currents during different phases of the spike (**Fig. 5A-D**). Since inward (depolarizing) current at voltages near spike threshold is critical for spike initiation (56; see above), we sought to identify which $Na_V$ contributes to that current. In the DIV0 model, $Na_V1.7$ dominated during onset of the first spike but all subsequent spikes were initiated by $Na_V1.8$ (**Fig. 5A,B**). This occurred because the small $Na_V1.7$ conductance at DIV0 quickly inactivated during the first spike and remained 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). However, inactivation of $Na_V1.7$ after the first spike was reflected by an increase in voltage threshold between the first and second spike, both in the model (**Fig. 5A**) and in experiments (**Fig.**

**5E**). This unexpected 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, $Na_V1.7$ and $Na_V1.3$ contributed to initiation of all spikes whereas $Na_V1.8$ was negligible **(Fig. 5C,D)**. 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 subtype contributes preferentially to a different spike phase depending on its voltage-dependency, but conductance density and inactivation status are both important. Indeed, an subtype's contribution can shift rapidly (because of channel inactivation) or slowly (because of gene expression changes).

***Control of 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 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. 57), these results suggest that translational regulation is crucial, though membrane trafficking and other downstream processes likely also contribute (58, 59).

***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 (60-63). 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. Inflammation caused nociceptors to become much more variable in their reliance of specific $Na_V$ subtypes (**Fig. 7A**). Despite this variability, inhibiting $Na_V1.7$ with PF-71 converted a significantly higher proportion of neurons to transient spiking after CFA (42%) than in control neurons (0%) (**Fig. 7B, left**); subsequent application of PF-24 to inhibit $Na_V1.8$ converted just 14% of CFA neurons to transient spiking vs 88% of control neurons (**Fig 7B, right**). 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. Consistent with this, epigenetic repression of $Na_V1.7$ prevents/reverses hypersensitivity in inflamed and neuropathic mice without causing hyposensitivity in naïve mice (64). 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 (65).

## 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.

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 (66, 67). 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 (68-70). Degeneracy also enables pathological changes in different ion channels to produce equivalent hyperexcitability (71). 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 (71, 72). Similar interchangeability is evident for the burst firing of Purkinje neurons (73).

Degeneracy also exists at the circuit level (74, 75), where it allows differences in the intrinsic excitability of component neurons to be offset (and effectively hidden) by differences in synaptic weights (76). Relevant for pain processing, the spinal dorsal horn circuit can achieve similar output using different synaptic weight combinations (77); 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 (78), but when inhibition is incompensably compromised, the underlying cause necessitates different interventions (79). 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 $Na_V1.7$ (80)) are responsible for mechanical allodynia under neuropathic conditions (81-84), 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, 85). 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 (86, 87). 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 $Na_V1.7$ (88). 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, 55). 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 (89) 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).

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.

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 (90). 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 (91). 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).

***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 (92). 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 where 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.

***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 RNA. Non-RT mRNA was also used as a negative control to exclude contamination from genomic RNA. 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) were 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 (93) . 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: $g_{\mathrm{Nav1.3}}$, $g_{\mathrm{Nav1.7}}$, $g_{\mathrm{Nav1.8}}$, $g_{\mathrm{Kdr}}$, $g_{\mathrm{M}}$, $g_{\mathrm{AHP}}$ and $g_{\mathrm{Leak}}$. 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 $\bar{g}_{\mathrm{Nav1.3}}$, $\bar{g}_{\mathrm{Nav1.7}}$ and $\bar{g}_{\mathrm{Nav1.8}}$, respectively, as reported in the figures. Channel noise was added as Ornstein-Uhlenbeck process with $\mu_{\mathrm{noise}}$= 0 $\mu\mathrm{A/cm^2}$, $\sigma_{\mathrm{noise}}$= 0.05 $\mu\mathrm{A/cm^2}$, and $\tau$ = 5 ms. All computer code is available at ModelDB (http://modeldb.yale.edu/267560; password: excitability).

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

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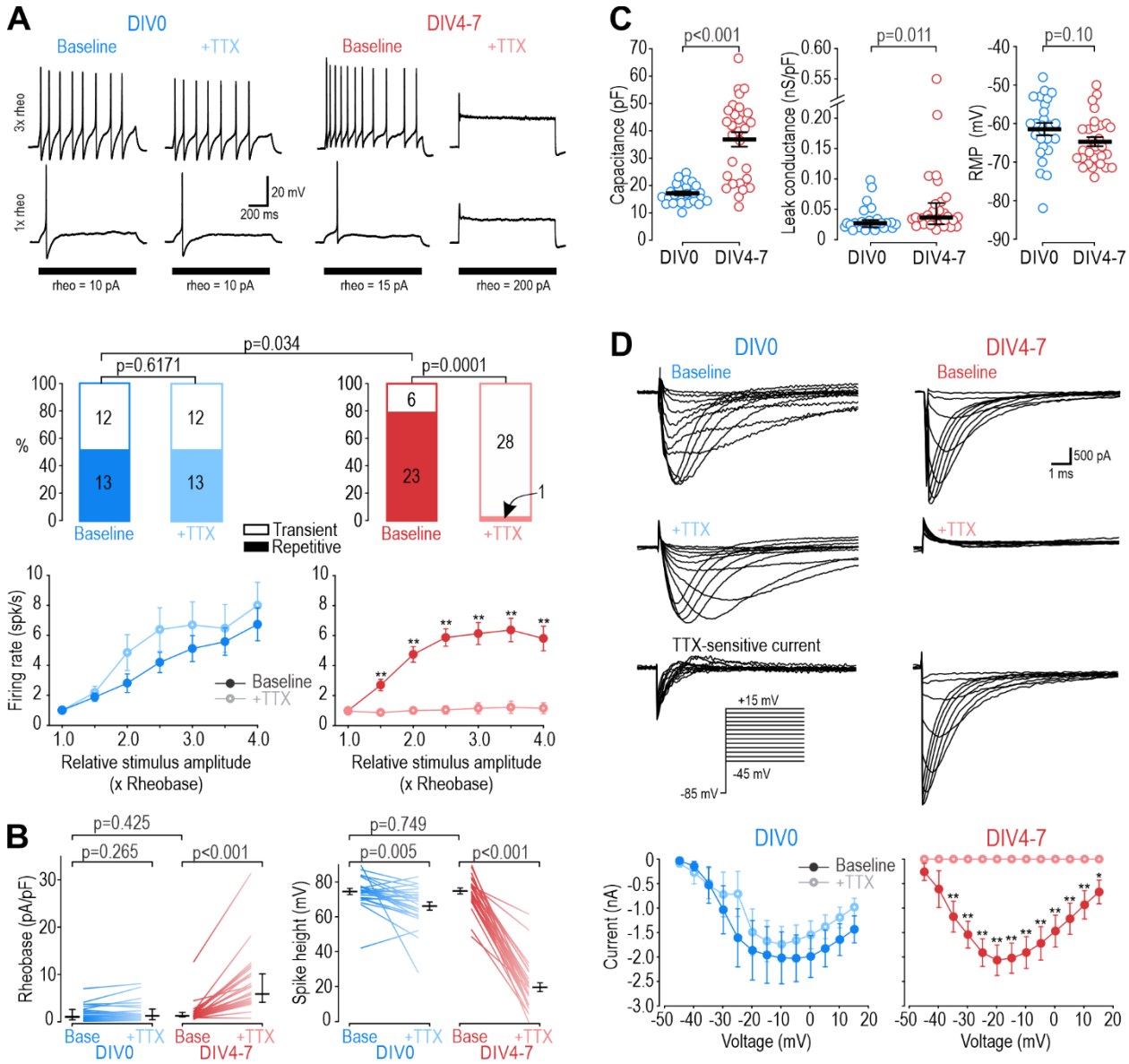


**Figure 1. Different $Na_V$ subtypes produce equivalent excitability at different days in vitro (DIV).**

**(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 ($\chi^2$=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 ($\chi^2$=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.

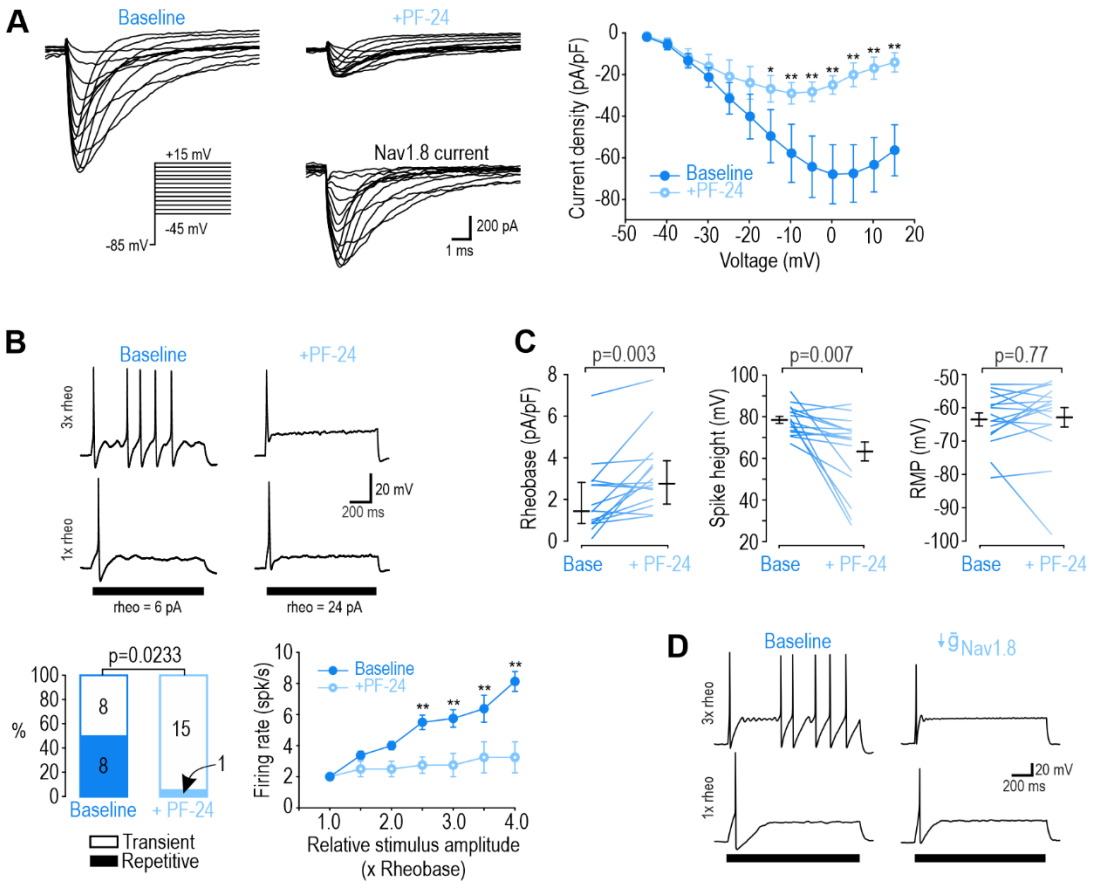


**Figure 2. $Na_V1.8$ is necessary for repetitive spiking at DIV0.**

**(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). Another $Na_V1.8$ inhibitor, A-803467, had a similar effect (see Fig. 2 – figure supplement 1). **(B)** PF-24 significantly altered spiking pattern ($\chi^2$=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 $Na_V1.8$ ($\bar{g}_{\mathrm{Nav1.8}}$= 4mS/cm2). *, p<0.05; **; p<0.01; Student-Newman-Keuls post-hoc tests in A and B.

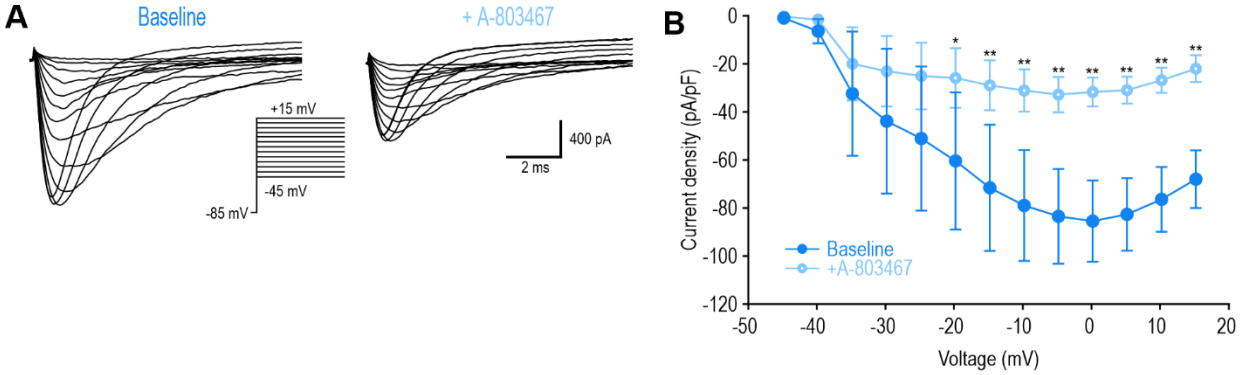


**Figure 2 – figure supplement 1. Inhibiting $Na_V1.8$ at DIV0 with A-803467 had the same effect as PF-24.**

**(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.

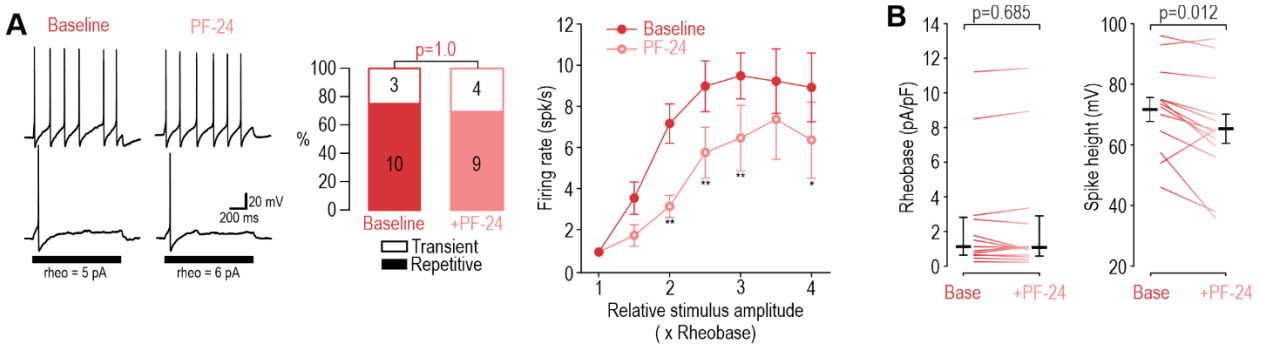


**Figure 2 – figure supplement 2. Inhibiting $Na_V1.8$ with PF-24 at DIV4-7 had negligible effects.**

**(A)** Inhibiting $Na_v1.8$ with PF-24 (1 µM) did not affect spiking pattern ($\chi^2$ =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.

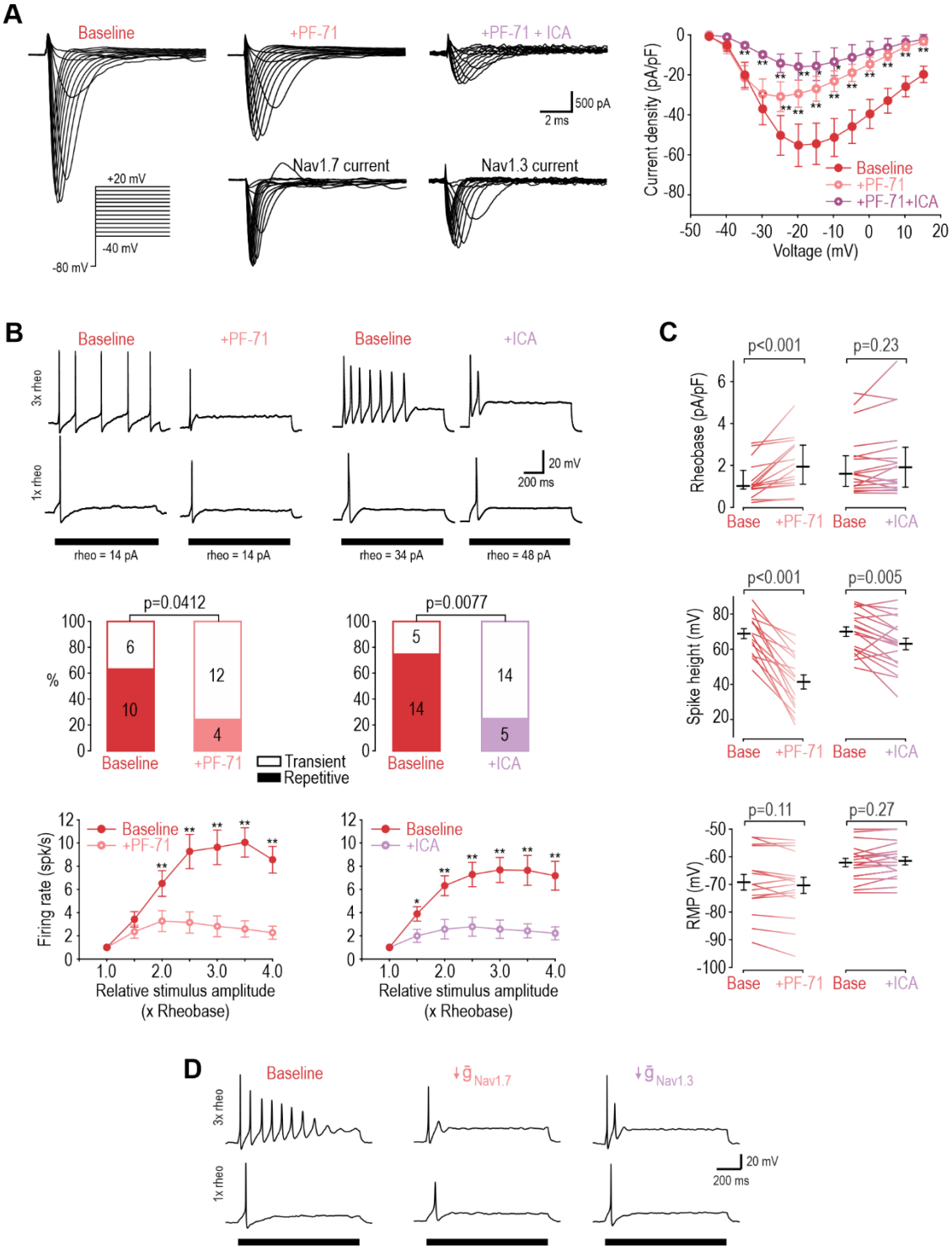


**Figure 3. $Na_V1.3$ and $Na_V1.7$ are necessary for repetitive spiking at DIV4-7.**

**(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). **(B)** PF-71 and ICA both significantly altered spiking

pattern ($\chi^2$=4.17, p=0.041 and $\chi^2$ =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 $Na_V1.7$ ($\bar{g}_{\mathrm{Nav1.7}}$= 10.5 mS/cm$^2$). ICA effect was simulated as a 90% reduction in $Na_V1.3$ ($\bar{g}_{\mathrm{Nav1.3}}$= 0.035 mS/cm$^2$). *, p<0.05; **, p<0.01; Student-Newman-Keuls post-hoc tests in A and B.

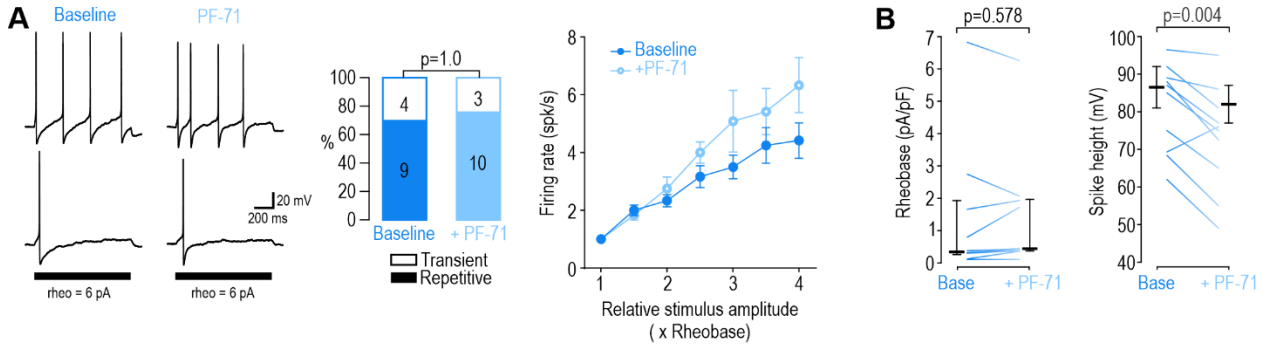


**Figure 3 – figure supplement 1. Inhibiting $Na_V1.7$ at DIV0 had negligible effects.**

**(A)** Inhibiting $Na_V1.7$ with PF-71 (30 nM) did not alter spiking pattern ($\chi^2$=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).

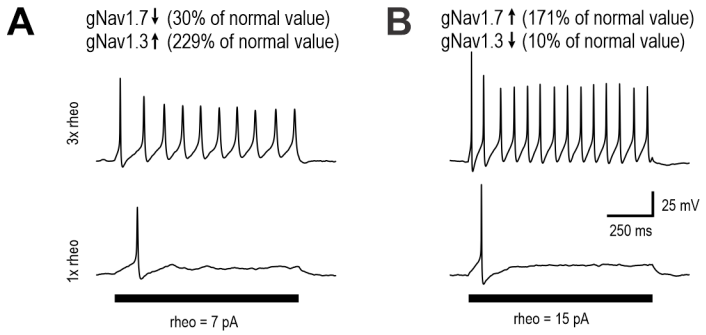


**Figure 3 – figure supplement 2. $Na_V1.7$ and $Na_V1.3$ currents can compensate for each other.**

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

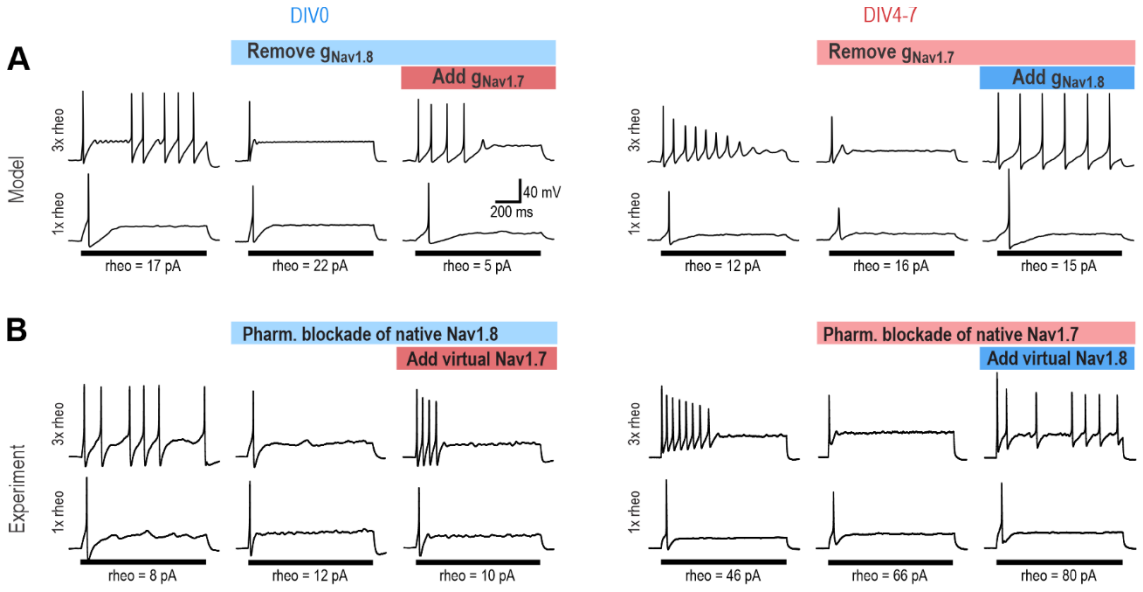


**Figure 4. $Na_V1.7$ and $Na_V1.8$ are each sufficient to produce repetitive spiking in DIV0 and DIV4-7 neurons. (A)** The computational model predicts that the $Na_V1.8$ conductance, which is "necessary" for repetitive spiking at DIV0 can, in principle, be replaced by $Na_V1.7$ (left), and vice versa 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.

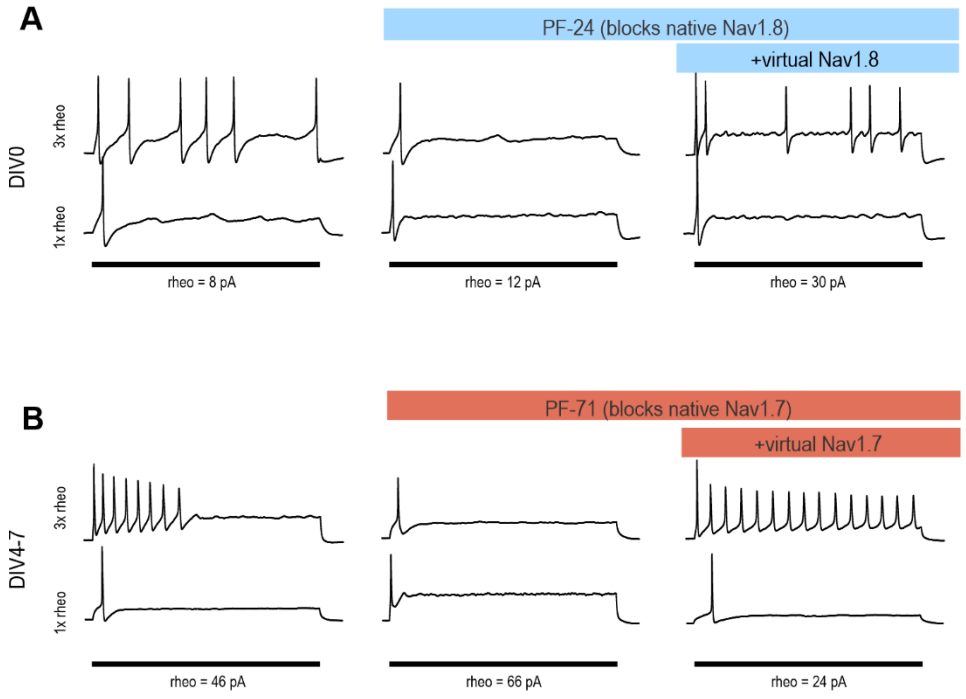


**Figure 4 – figure supplement 1. Virtual conductances restored repetitive spiking after pharmacological inhibition of the corresponding native conductance had converted the neuron to transient spiking.**

**(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.

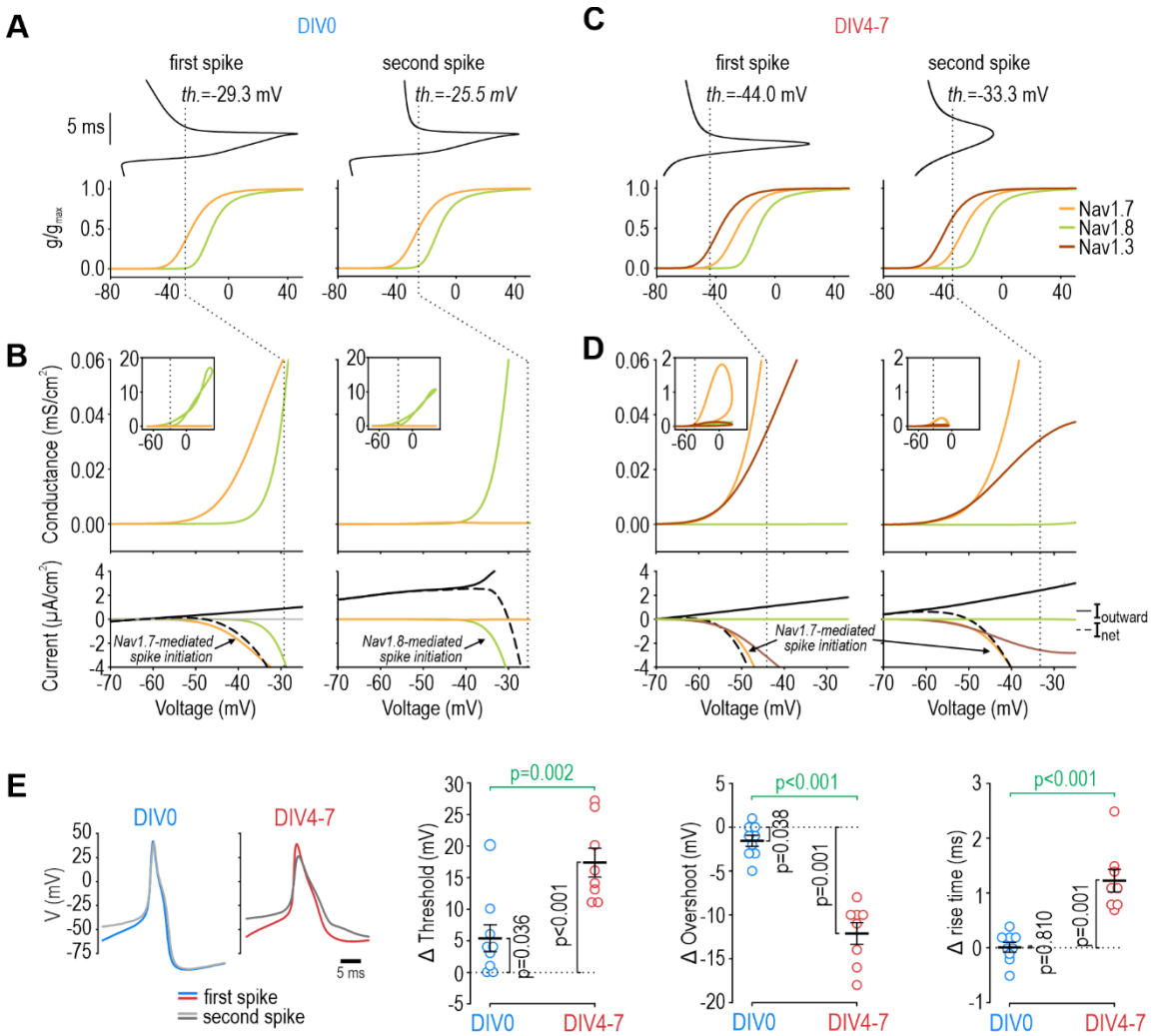


**Figure 5. Contribution of $Na_V1.7$ and $Na_V1.8$ to spike initiation in DIV0 and DIV4-7 neurons.**

**(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 1$^{st}$ and 2$^{nd}$ 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 DIV1 (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.

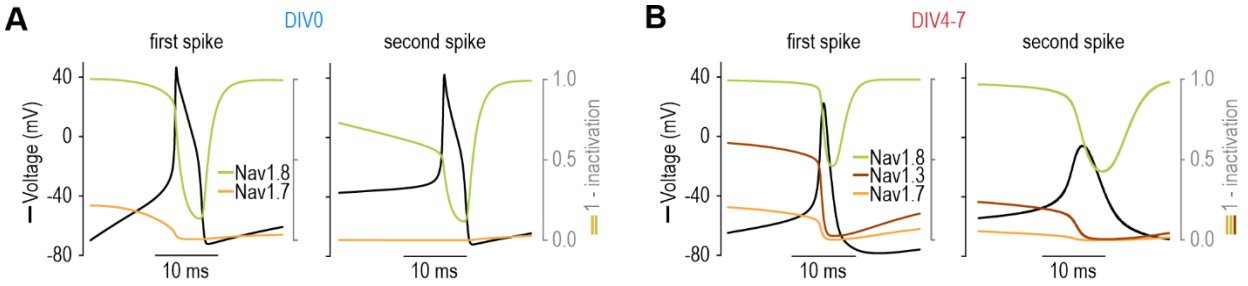


**Figure 5 – figure supplement 1. Channel inactivation affects $Na_V$ subtype contribution on short timescale.** **(A)** In the DIV0 model, $Na_V1.7$ contributed to the first spike but its inactivation meant that all subsequent spikes relied exclusively on $Na_V1.8$. **(B)** In the DIV4-7 model, despite 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.

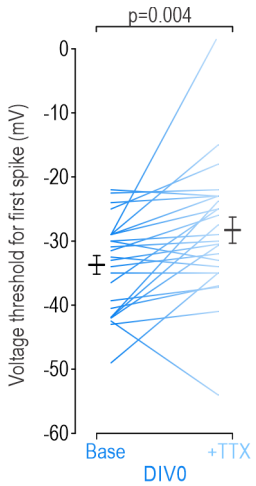


**Figure 5 – figure supplement 2. Effect of TTX on voltage threshold in DIV0 neurons.**
Despite TTX having negligible effects in DIV0 neurons according to our initial analysis (see Fig. 1), simulation results in Fig. 5A,B predicted that the first spike was nonetheless initiated by $Na_V1.7$. By extension, 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.

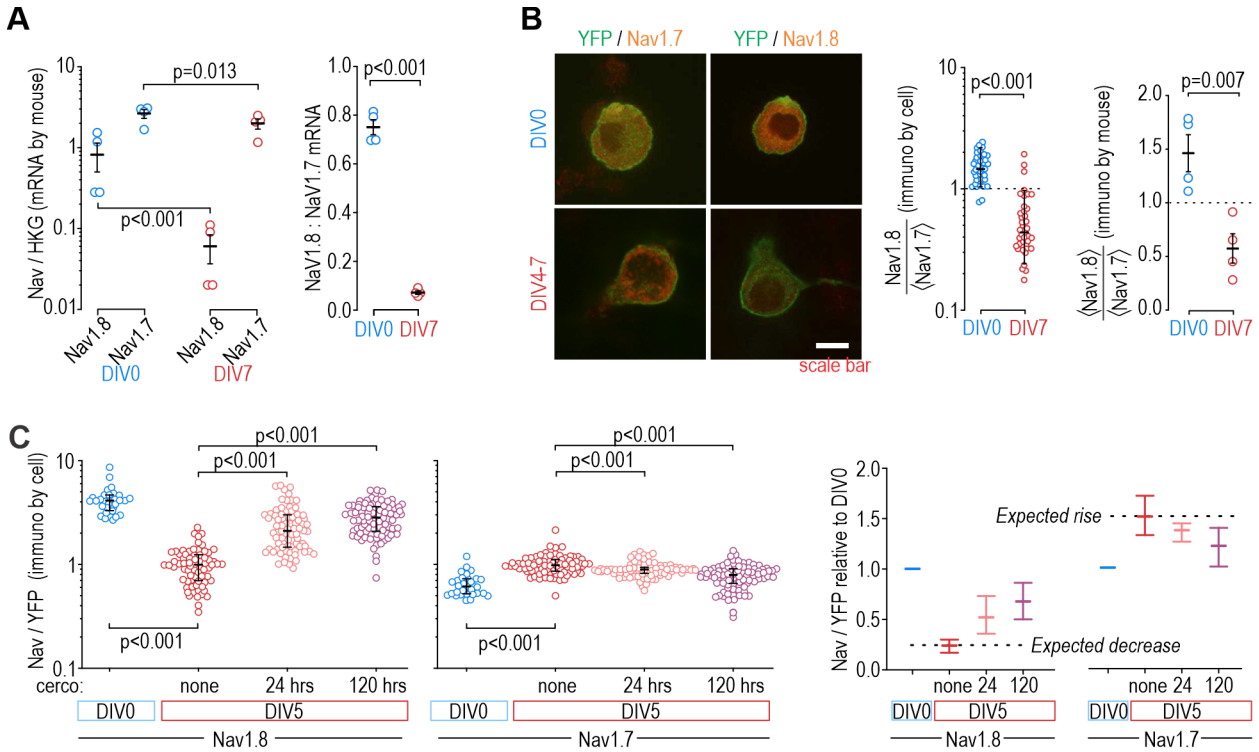


**Figure 6. Protein levels, but not mRNA, reflect functional contributions of $Na_V$ subtypes at DIV0 and DIV7.** **(A)** Both $Na_V1.8$ and $Na_V1.7$ mRNA levels (relative to a housekeeping gene (HKG), see Methods) decreased significantly between DIV0 and DIV4-7 (factor 1: time, $F_{1,12}$=56.677, p<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 the 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). Panel on the right shows data normalized to baseline (DIV0) to emphasize relative changes.

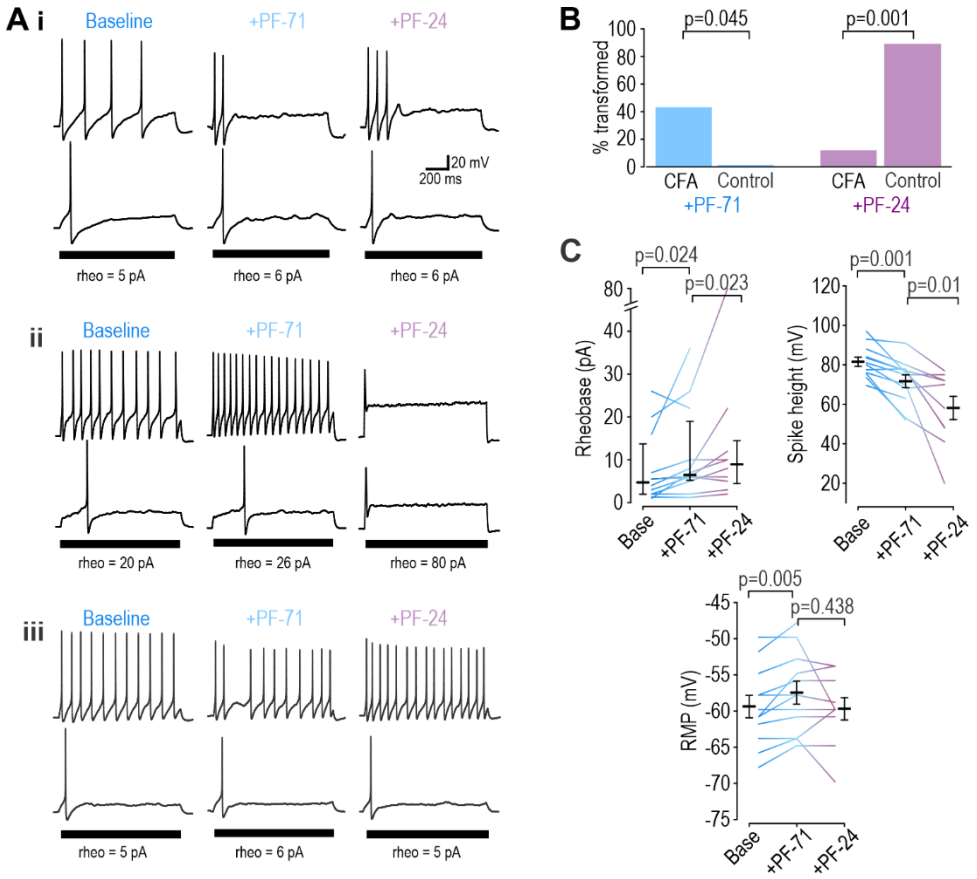


**Figure 7. Inflammation alters $Na_V$ subtype contribution to nociceptor excitability.**

(**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 neuron, 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).

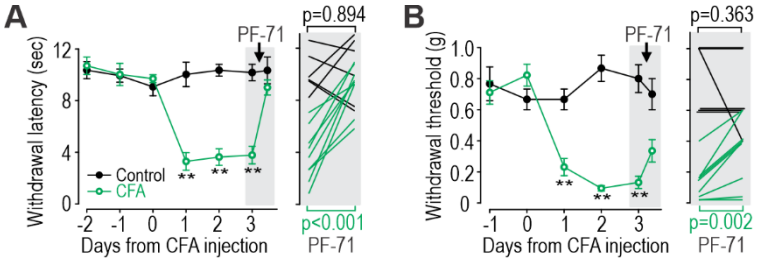


**Figure 8. Inflammation-induced change in $Na_V$ subtype contribution impacts analgesic efficacy of PF-71.** **(A)** CFA significantly increased thermal sensitivity ($F_{5,65}$=19.556, p<0.001, two-way RM ANOVA). PF-71 significantly decreased thermal sensitivity in mice injected three days prior with 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.

***Supplementary Table 1. Model data before and after channel "inhibition"***

| | 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 2. Reagents***

| Reference term | Description | Concentration used | Source |
|---|---|---|---|
| **TTX** | Tetrodotoxin citrate | 100 µM | Alomone, Israel |
| **PF-24** | PF-01247324 | 1 µM | Sigma, USA |
| **PF-71** | PF-05089771 | 30 nM | Alomone, Israel |
| **ICA** | ICA-121431 | 1 µM | Tocris, USA |
| **Papain** | Papain lyophilized power | 27u/ml | Worthington |
| **Collagenase** | Collagenase type II | 4mg/ml | Worthington |
| **Dispase II** | Dispase type II | 4.7mg/ml | Sigma |

***Supplementary Table 3. Primers***

| | 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. Model equations***

| Channel Model | Equations |
|---|---|
| $Na_V1.7$(86) | $I_{NaV1.7} = \bar{g}_{NaV1.7} m^3 h (V - E_{Na})$<br>$\dot{m} = \alpha_m(1-m) - m\beta_m$ $\quad$ $\dot{h} = \alpha_h(1-h) - h\beta_h$<br>$\alpha_m = 10.22 - \frac{10.22}{1+\exp\left(\frac{V+7.19}{15.43}\right)}$ $\quad$ $\alpha_h = \frac{0.0744}{1+\exp\left(\frac{V+99.76}{11.07}\right)}$<br>$\beta_m = \frac{23.76}{1+\exp\left(\frac{V+70.37}{14.53}\right)}$ $\quad$ $\beta_h = 2.54 - \frac{2.54}{1+\exp\left(\frac{V+7.8}{10.68}\right)}$ |
| $Na_V1.3$* | $I_{NaV1.3} = \bar{g}_{NaV1.3} m^3 h (V - E_{Na})$<br>$\dot{m} = \alpha_m(1-m) - m\beta_m$ $\quad$ $\dot{h} = \alpha_h(1-h) - h\beta_h$<br>$\alpha_m = 10.22 - \frac{10.22}{1+\exp\left(\frac{V+7.19+12}{15.43}\right)}$ $\quad$ $\alpha_h = \frac{0.0744}{1+\exp\left(\frac{V+99.76}{11.07}\right)}$ |

| | |
|---|---|
| | $\beta_m = \frac{23.76}{1+\exp\left(\frac{V+70.37+12}{14.53}\right)}$ $\beta_h = 2.54 - \frac{2.54}{1+\exp\left(\frac{V+7.8}{10.68}\right)}$ |
| $Na_V1.8$(87) | $I_{NaV1.8} = \bar{g}_{NaV1.8} m^3 h (V - E_{Na})$<br>$\dot{m} = \alpha_m(1-m) - m\beta_m$ $\dot{h} = \alpha_h(1-h) - h\beta_h$<br>$\alpha_m = 7.21 - \frac{7.21}{1+\exp\left(\frac{V-0.063}{7.86}\right)}$ $\alpha_h = 0.003 + \frac{1.63}{1+\exp\left(\frac{V+68.5}{10.01}\right)}$<br>$\beta_m = \frac{7.4}{1+\exp\left(\frac{V+53.06}{19.34}\right)}$ $\beta_h = 0.81 - \frac{0.81}{1+\exp\left(\frac{V-11.44}{13.12}\right)}$ |
| $K_M$(88) | $I_{KM} = \bar{g}_{KM} n (V - E_K)$<br>$\dot{n} = \frac{n_\infty - n}{\tau_n}$<br>$n_\infty = \frac{1000}{1+e^{-\frac{V+35}{5}}}$ $\tau_n = \frac{1000}{3.3e^{V+\frac{35}{10}} + e^{-\frac{V+35}{10}}}$ |
| $K_{dr}$(88) | $I_{KA} = \bar{g}_{KA} n^3 l (V - E_K)$<br>$\dot{n} = \alpha_m(1-m) - m\beta_m$ $\dot{l} = \alpha_h(1-h) - h\beta_h$<br>$\alpha_n = \frac{e^{(-5\times10^{-3}\cdot(V+32)\cdot 9.648\times10^4)}}{2562.35}$ $\alpha_l = \frac{e^{(2\times10^{-3}\cdot(V+61)\cdot 9.648\times10^4)}}{2562.35}$<br>$\beta_n = \frac{e^{(-2\times10^{-3}\cdot(V+32)\cdot 9.648\times10^4)}}{2562.35}$ $\beta_l = \frac{e^{(-2\times10^{-3}\cdot(V+32)\cdot 9.648\times10^4)}}{2562.35}$ |
| AHP** | $I_{AHP} = \bar{g}_{AHP} z (V - E_K)$ $\dot{z} = \frac{1}{1+e^{5-V/4}} - \frac{z}{100}$ |

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

| | Conductance densities at baseline (mS/cm$^2$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | $\bar{g}_{Nav1.3}$ | $\bar{g}_{Nav1.7}$ | $\bar{g}_{Nav1.8}$ | $\bar{g}_{Kdr}$ | $\bar{g}_{M}$ | $\bar{g}_{AHP}$ | $\bar{g}_{Leak}$ |
| 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 |