Optimising a difficult Nav1.8 cell line assay for automated patch clamp screening

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Introduction

The TTX-resistant sodium channel Nav1.8 is expressed in peripheral sensory nociceptors and is implicated in a range of inflammatory and visceral pain conditions such as irritable bowel syndrome (IBS)[1]. Nav1.8 channel function in sensory neurons changes after injury or inflammation, with a redistribution from the soma to axons and upregulated activity through inflammatory mediators and signalling pathways[2]. This is thought to underlie increased neuronal excitability and a greater role of Nav1.8 currents in persistent, repetitive and ectopic action potential firing in inflammatory and visceral pain. Several gain-of-function mutations in Nav1.8 have also been detected in human patients suffering from small fibre neuropathy (SFN)[3], offering similar genetic target validation and clinical population for personalised medicine approaches seen with Nav1.7 mutations in primary erythmelalgia, paroxysmal extreme pain and SFN patients.

Although Nav1.8 is evolutionarily and structurally distinct from the major family of TTX-sensitive (TTX-S) Nav1.x channels expressed in nociceptors, preclinical treatments have utilised non-selective Nav antagonists like anti-convulsants (Amitriptyline), anaesthetics (Lidocaine) and anti-epileptics (Lamotrigine) to reduce pain behaviour. In the clinic these compounds are poorly tolerated due to serious side effects and difficult dose titration which narrow their therapeutic index. This has prompted recent efforts to discover selective Nav1.8 antagonists but these have either failed to translate to the clinic (e.g. Abbott, Pfizer-Icagen) or proceeded due to a mixed Nav1.8/Nav1.7 profile (Xenon). A successful drug discovery screening cascade for Nav1.8 requires a robust cell line for this difficult-to-express target that is suitable for automated patch clamp assays designed to discover potent, selective and drug-like state-dependent ligands from high throughput screens (HTS) and focused MedChem SAR campaigns.

Methods & Materials

Tissue culture

HEK-293 and ND7-23 cell lines stably expressing exogenous human and rat $Na_v1.8$ and β1 subunits, were initially cultured according to commercial suppliers protocols. HEK cells were grown in DMEM/F12 media supplemented with non-essential amino acids (NEA) and 10% FBS, while ND7-23 cells were grown in DMEM media supplemented with 10% FBS. 'Mother' T-175 flasks were routinely passaged every 2-3 days to keep cell confluency <80%. Flasks destined for patch clamp experiments were typically grown for 2-5 days to enable application of a variety of standard and novel treatments to increase expression of Nav1.8 channels at the cell surface. We also compared several cell dissociation reagents (enzyme and enzyme-free) and cell preparation methods to optimise cell viability, "patchability", and obtain single cell suspensions suitable for APC.

Automated patch-clamp (APC) electrophysiology

Both $Na_v1.8$ cell lines were validated biophysically and pharmacologically on the PatchLiner (Nanion, Germany) and QPatch platforms (Sophion, Denmark). We used standard single hole chips (~2.5MΩ resistance), as well as multi-hole formats on PatchLiner (2-8 hole) and QPatch (10 hole); both sum amplitudes and divide seal resistance from single sites in multi-hole wells.

Solutions: Internal solution for both APC platforms contained (in mM): 120 CsF, 10 NaCl, 10 HEPES, 5 Na_2-ATP, 10 EGTA, 1 $MgCl_2$; pH 7.2, ~290mOsm. External solution contained (in mM): 140 NaCl, 5 KCl, 10 Glucose, 10 HEPES, 2 $MgCl_2$ and 1 $CaCl_2$; pH 7.4, ~310mOsm.

Voltage protocols: All recordings were in conventional whole cell configuration achieved through a combination of suction and voltage zaps. Nav currents were elicited from a negative holding potential (typically Vh -100mV) with brief (20-50ms) activating test pulses. Nav1.8 current amplitude was monitored using test pulses to +20mV applied every 10-15s. Current-voltage (IV) curves were constructed by applying test pulses in 5-10mV increments every 30-60s from -60mV, while steady-state inactivation (Vh) curves used incrementing conditioning prepulses every 60s from -120mV. Compound screening utilised a 4 pulse protocol delivered every 15s to assess resting (Peak1), paired-pulse (P2) and inactivated state inhibition (P3), followed by recovery (P4). The 5s conditioning prepulse before Peak 3 was optimised to achieve ~50% inactivation for each cell line on each platform on each assay day (pre-defined across all cells on QPatch, manually or automatically adjusted for each cell on PatchLiner). Series resistance (4-15MΩ) was compensated by 65-85% and leak subtraction calculated using a P/n protocol; the latter could be toggled on or off in both APC software programs.

Data analysis: Peak current amplitude during the activating test step (1-20% duration) was measured by placing cursors in HEKA PatchMaster or QPatch Assay Software. IV curves were plotted as peak current *vs.* test potential, while Vh steady-state inactivation curves plotted test pulse amplitude *vs.* pre-pulse conditioning voltage (normalised to maximum current or -100mV).

Compound screening

Vehicle (0.1% DMSO) was applied to the cells to achieve a stable control recording (4min) and then compound potency was determined from either single or cumulative applications of test compound (5-10μL or 30-50μL on QPatch and PatchLiner, respectively). We typically applied two bolus additions per concentration to obtain 3pt mini-IC_{50} or 4pt IC_{50} estimates of compound potency. Concentration response curves (four parameter logistic curve) were fitted to %inhibition data using Prism (GraphPad) from which IC_{50} (50% inhibitory concentration) and Hill coefficients were determined (hill slope constrained to $0.5>n_h<2.0$). All %inhibition and IC_{50} data is N ≥ 3.

Results

1. Optimising expression of a hNav1.8 cell line

Several commercial hNav1.8 stable cell lines were evaluated before we selected the best expressor for further assay optimisation. Nav1.8 is notoriously difficult to express in heterologous systems and most groups report peak current amplitudes of only 500-1000pA. This is insufficient for screening purposes as:

- Current amplitude is frequently reduced in cell suspensions used for APC
- Nav1.x screening assays employ a 30-50% inactivated state protocol

We explored many mechanisms to boost both current amplitude and expression rate.

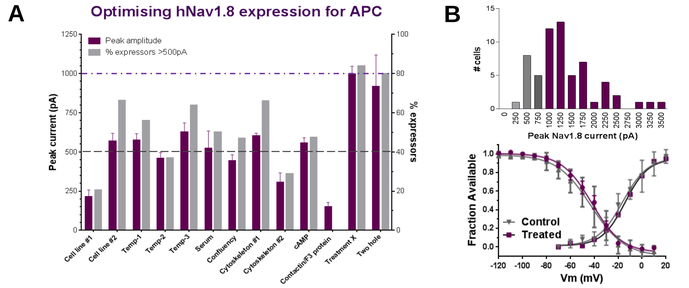

Figure 1: **Tissue culture & assay conditions used to modulate Nav1.8 expression**
A: Lines denote minimum (500pA) and desired (1nA) current & % expressors on PL.
B: Preferred treatment increases Nav1.8 current (*upper*) without altering biophysics (*lower*).

2. PatchLiner single point screening assay for hNav1.8

Once expression was optimised we could build and validate Nav1.8 screening assays. The PatchLiner features continuous waste removal to enable large application volumes and washout suitable for single point screening. A 4 pulse voltage protocol was used with Pulse 3 measuring 50% inactivated state inhibition (**A**). The optimised assay screened 3,000 compounds over 2 months with consistent success (**B**).

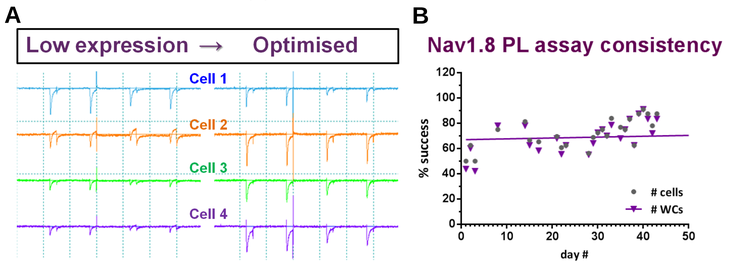

Figure 2: Optimised Nav1.8 expression and good "patchability" yields a successful assay
A: 4 pulse protocol current traces from control & optimised cells. (Scale: blue grid = 2nA).
B: Nav1.8 assay success rate measured each day as cells captured and whole cells converted.

3. QPatch hNav1.8 IC_{50} screening assay optimisation & validation

Nav1.8 current expression and assay success were reduced in single hole recordings on QPatch compared to PatchLiner, so a multi-hole "X-mode" assay was developed. Biophysics and reference pharmacology were similar to single hole recordings.

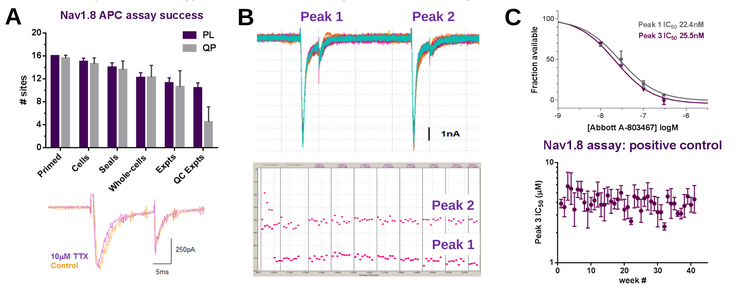

Figure 3: Improved Nav1.8 expression and assay consistency in QPatch multi-hole X-mode
A: Single hole assay shows good "patchability" but small TTX-resistant currents which fail QC.
B: Multi-hole currents are large & stable, enabling reliable state-dependent 4pt IC_{50} screening.
C: Pharmacological validation and long term stability of state-dependent Nav1.8 assay.

4. Optimising a rNav1.8 assay in ND7-23 neuroblastoma cell line

A commercial cell line expressing rat Nav1.8 was used to build an APC assay on PatchLiner (**A**) and QPatch (**B**) to measure species and sub-type selectivity. The ND7-23 cell background includes endogenous accessory subunits that aid exogenous rNav1.8 expression, as well as native rodent TTX-sensitive Nav1.x channels. We hoped to increase rNav1.8 over TTX-s levels.

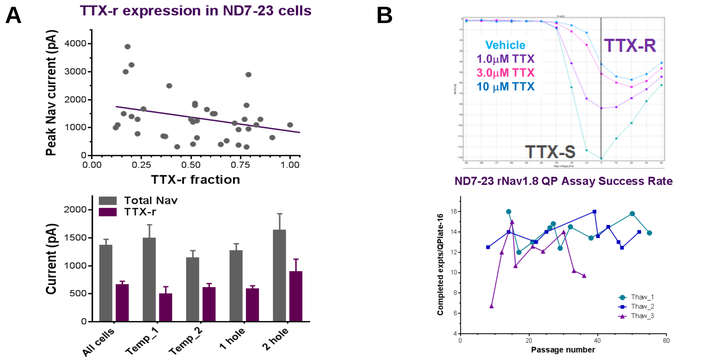

Figure 4: Characterisation & validation of APC assay for rNav1.8 channels in ND7-23 cells
A: Consistent expression of TTX-resistant component of total I_{Na} in ND7-23 cells on PatchLiner.
B: QPatch rNav1.8 TTX-r assay shows expected biophysics and consistent success rate.

5. Profiling endogenous TTX-s Nav1.x channels in ND7-23 cells

Molecular techniques (e.g. PCR, antibody binding) indicate Nav1.7 is the predominant species of TTX-sensitive channel in ND7-23 cells, and also reveal the presence of Nav1.2 and Nav1.3 or Nav1.6 subunits.[4,5] We determined the repertoire of *functional* Nav1.x TTX-s channels on the surface of ND7-23 cells by applying toxins and small molecules selective for Nav1.6 (4,9 anhydro TTX), Nav1.7 (Protoxin-II) and Nav1.3 (Icagen). Selective concentrations were chosen from QPatch IC_{50}'s determined against heterologous Nav1.3, Nav1.6 and Nav1.7 channels.

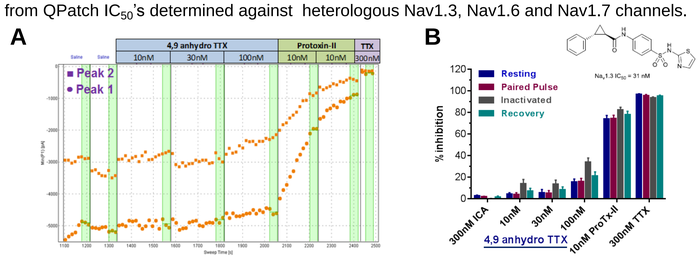

Figure 5: **Predominant expression of Nav1.7 >> Nav1.6 TTX-s channels in ND7-23 cells**
A: QPatch recording of whole cell I_{Na} showing sequential block by Nav1.6-selective doses of 4,9 anhydro TTX, Nav1.7-selective Protoxin (10nM) and complete inhibition by TTX (300nM)
B: Contributions from Nav1.3, Nav1.6 and Nav1.7 to state-dependent activity of TTX-s current.

Conclusions

- Metrion's expertise in ion channel cell line optimisation and assay development enabled the development of reliable automated patch clamp assays for both human and rat Nav1.8 channels, targets that are notoriously difficult to express.
- Assay optimisation achieved increases in both Nav1.8 peak amplitude (>1nA) and % expressors (>80%) while maintaining good "patchability" to enable efficient and consistent drug discovery screening.
- Multi-hole recording increased Nav1.8 current amplitude on both APC platforms. This was essential for a successful assay on the QPatch, but the benefit was outweighed by reduced success rate (QC completion) on the PatchLiner.

References

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