

Stability and reproducibility of hNa_v1.8 recordings on Nanion's SyncroPatch® 384PE

The electrophysiology team at Nanion Technologies GmbH, Munich, Germany. Cells were kindly provided by Charles River.



Summary

The Na_v1.8 gene (originally named PN3 or SNS; gene symbol SCN10A) encodes a voltage-gated sodium (Na_v) channel, selectively expressed in dorsal root ganglion (DRG) neurons^{1,2}. In contrast to the fast and rapidly inactivating TTX-sensitive channels, Na_v1.8 is TTX-resistant and exhibits slower kinetics with a depolarized voltage-dependence of activation and inactivation². hNa_v1.8 is an interesting drug target for inflammatory and neuropathic pain because modulation of this ion channel by inflammatory mediators appears to be a key mechanism of DRG nociceptor sensitization and activation³. Interestingly, the development of potent and selective Na_v1.8 inhibitors has shown promising results in reducing neuropathic pain in animal models and this has fueled interest in the search for selective Na_v1.8 inhibitors².

The bottleneck for drug discovery involving ion channels is often the electrophysiological assays. Nanion's SyncroPatch 384PE offers a high throughput gigaseal platform which records up to 384/768 experiments simultaneously which helps to address this problem. It enables the recording of high quality data with reliable pharmacology, and biophysical characterizations of the protein. Our results show current-voltage relationships consistent with published results⁴⁻⁶ and very stable recordings using multi-hole chips. Furthermore, we show activation of hNa_v1.8 from different states results in altered compound affinity. We demonstrate the suitability of Nanion's SyncroPatch 384PE for high throughput screening of hNa_v1.8.

Results

Figure 1 shows current responses to increasing voltage steps for an exemplar CHO cell expressing hNa_v1.8 and the corresponding current-voltage plot (normalized to the peak amplitude for each cell) for an average of 380 cells. hNa_v1.8-mediated currents started to activate at about -40 mV and peak response was elicited between 10 and 20 mV. The data was fitted using a Boltzmann equation revealing a V_{half} of activation of -2.7 mV ($n = 380$), in good agreement with the range found in the literature⁴⁻⁶.

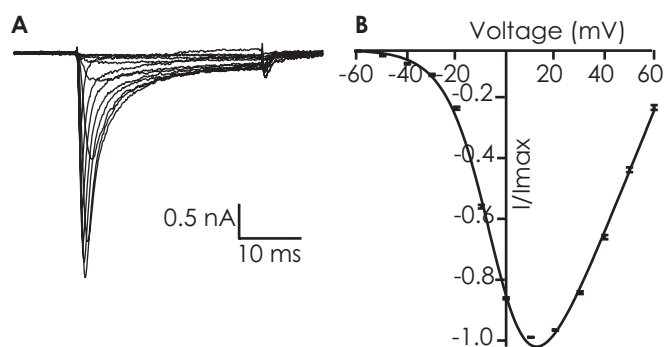


Figure 1: **A** Raw traces from an exemplar cell expressing hNa_v1.8 recorded on the SyncroPatch® 384PE. Shown are current responses to increasing voltage steps from -60 to +60 mV. **B** Current-voltage plot for an average of 380 cells. Shown are mean of peak amplitudes normalized to the maximum of each cell ± S.E.M.

Application Note

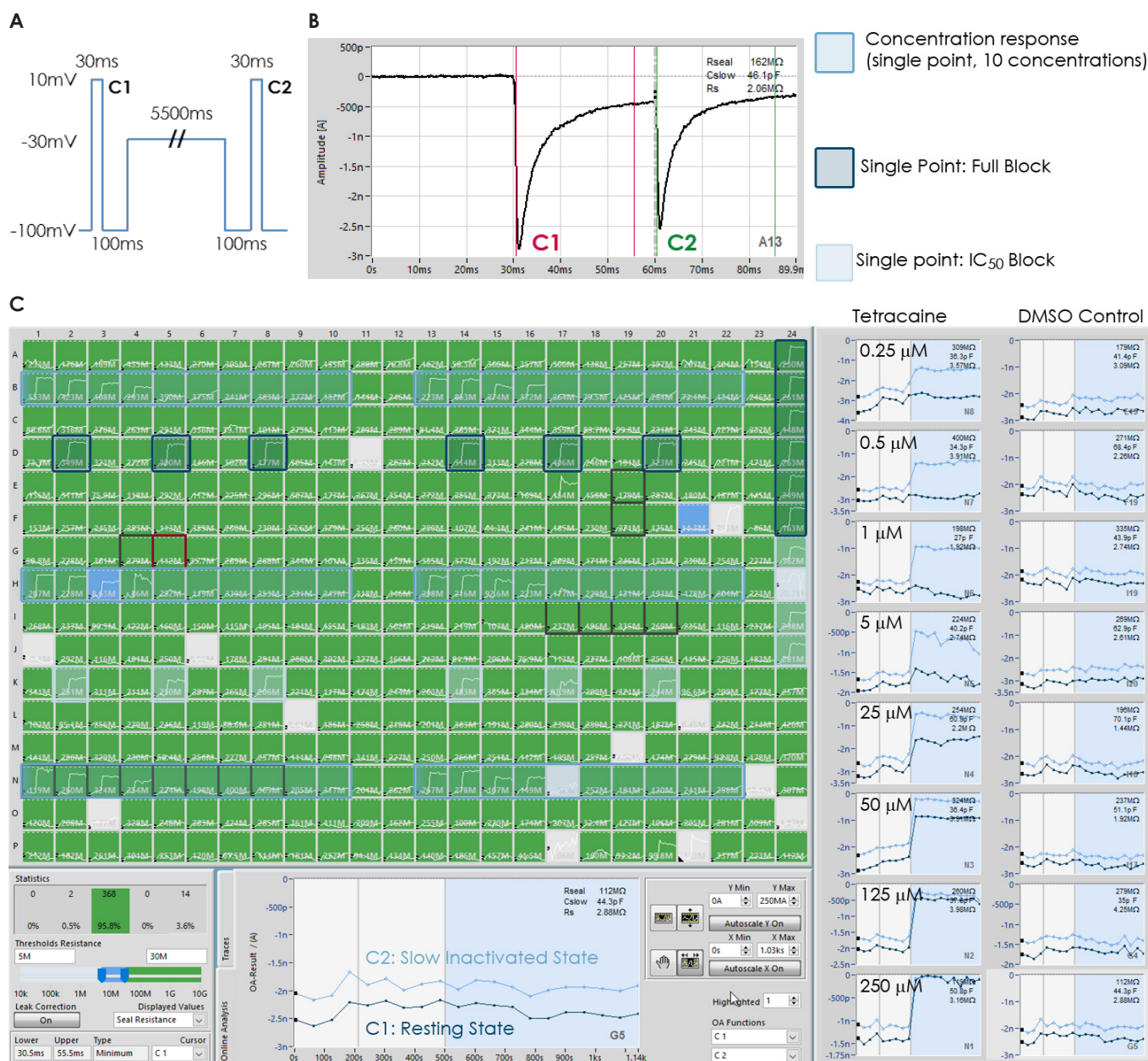


Figure 2: A Voltage protocol for the pharmacology experiments. C1 represents the peak current from the resting state and C2 represents peak current from the slow inactivated state. **B** Traces from an exemplar cell to the voltage protocol shown in **A**. Cursors for C1 (red) and C2 (green) are shown. **C** Screenshot of the data acquisition software for the SyncroPatch 384PE showing the time course of current amplitudes applying the two-step pulse protocol shown in **A**. During this experiment, 302 of the wells received vehicle (0.25% DMSO) to monitor change in current over time. On the same plate, a 10-point concentration response curve (each well received 1 concentration) for tetracaine was performed 6 times (shaded light blue rectangles), and two further controls: single points for IC₅₀ concentration (very light blue, 25 μM Tetracaine) and Full Block (dark blue, 250 μM Tetracaine) were used. The responses to tetracaine and DMSO only are highlighted on the right for 16 selected wells and for one at the bottom (DMSO control) in order to monitor the experiment. Compound incubation time was 10 minutes. This highlights not only the stability of the recordings, but also the flexibility of experimental design using 384-well compound plates.

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Na_v1.8 has traditionally been a difficult target to study due to difficulty in expression in mammalian cell lines⁵. Using the SyncroPatch 384PE and a CHO cell line stably expressing hNa_v1.8, we were able to reliably record hNa_v1.8-mediated currents with activation parameters consistent with hNa_v1.8⁴⁻⁶ at a high success rate (99% for completed current-voltage plots; Figure 1). However, due to low current amplitudes using single hole chips (>80% of cells exhibited peak current at 10 mV of <300 pA), multi-hole (9X) chips were used for the experiments which increased the success rate to >97% of cells exhibiting a peak amplitude of -1 to -3 nA.

In order to assess the potency of compounds on the slow inactivated state of hNa_v1.8 versus the resting

state, the voltage step protocol shown in Figure 2A was devised. Figure 2B shows a recording from an exemplar cell using this two-step voltage protocol. Using multi-hole (9X) chips, the peak current amplitude at C2 (slow inactivated phase) was consistently in the range of -1 to -3 nA, suitable for screening compounds on this ion channel. Figure 2C shows a screenshot of a typical experiment highlighting the flexibility in experimental design. On the plate, negative, positive, and single point IC₅₀ controls were performed along with concentration response curves.

Figure 3 shows 3 concentration response curves calculated from different plates on different days. The concentration response curves were calculated for

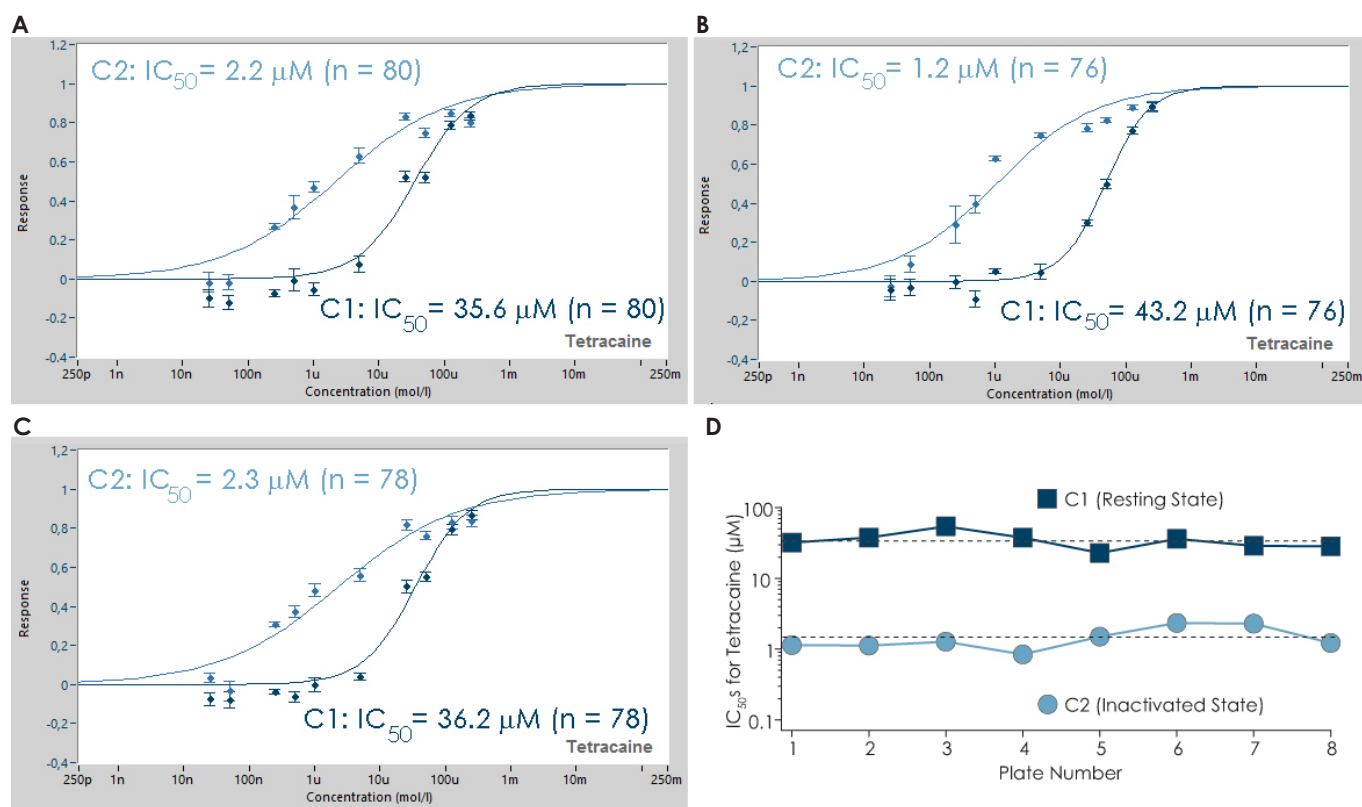


Figure 3: Shown are 3 concentration response curves (A-C) calculated on different days using different plates and cell preparations. The peak current amplitudes at C1 (resting state) and C2 (slow inactivated state; see Figure 2) were normalized to control and are shown. A Hill equation was fitted and the calculated IC₅₀ values at C1 (dark blue) and C2 (light blue) are shown for each plot. The IC₅₀ is shifted at least 15-times to more potent values when measured at C2 vs. C1 under these conditions. **D** The graph shows IC₅₀ values obtained for tetracaine on different days and cell preparations for C1 (dark blue squares) and C2 (light blue circles). On average, IC₅₀s were 34.8 ± 3.3 μM (n = 8 plates) for C1 and 1.5 ± 0.2 μM (n = 8 plates) for C2 as indicated by the dashed lines. Using these conditions, the shift was always larger than 15-fold and, importantly, the IC₅₀ values vary very little across the plates.

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C1 and C2 for each well and the average concentration response curves were constructed for each plate. Importantly, the IC_{50} values for tetracaine at C2, the slow inactivated state, were at least 15 times more potent than at C1 under these conditions. The IC_{50} values calculated for C1 and C2 were consistent across plates. This is also shown in Figure 3D where the IC_{50} values are plotted for 8 different plates. The values vary very little from the mean which is shown by the dashed line.

Importantly, current amplitudes were consistent across wells, plates and on different days using different cell preparations. Figure 4A and B show that normalized peak current values for C1 and C2, respectively, are consistent across plates when experiments are performed on different days using different cell

preparations. Figure 4C shows the spread of amplitudes (normalized to control at the start of the experiment) in negative (0.25% DMSO) or positive (250 μ M tetracaine) control conditions. The Z factor, a statistical parameter for evaluation and validation of high throughput screening (HTS) data⁷, was calculated and revealed a Z' value of 0.52 for this assay with respect to the positive and negative controls, proving that this is an excellent assay for HTS⁷ of hNa_v1.8. Figure 4D shows consistency of success rates at the end of the experiment (using quality control criteria $I > 200$ pA at the start of the experiment); full block using positive control; RSeal > 30 M Ω at the end of the experiment). Success rates for completed experiments were typically $> 90\%$ and Z values > 0.5 .

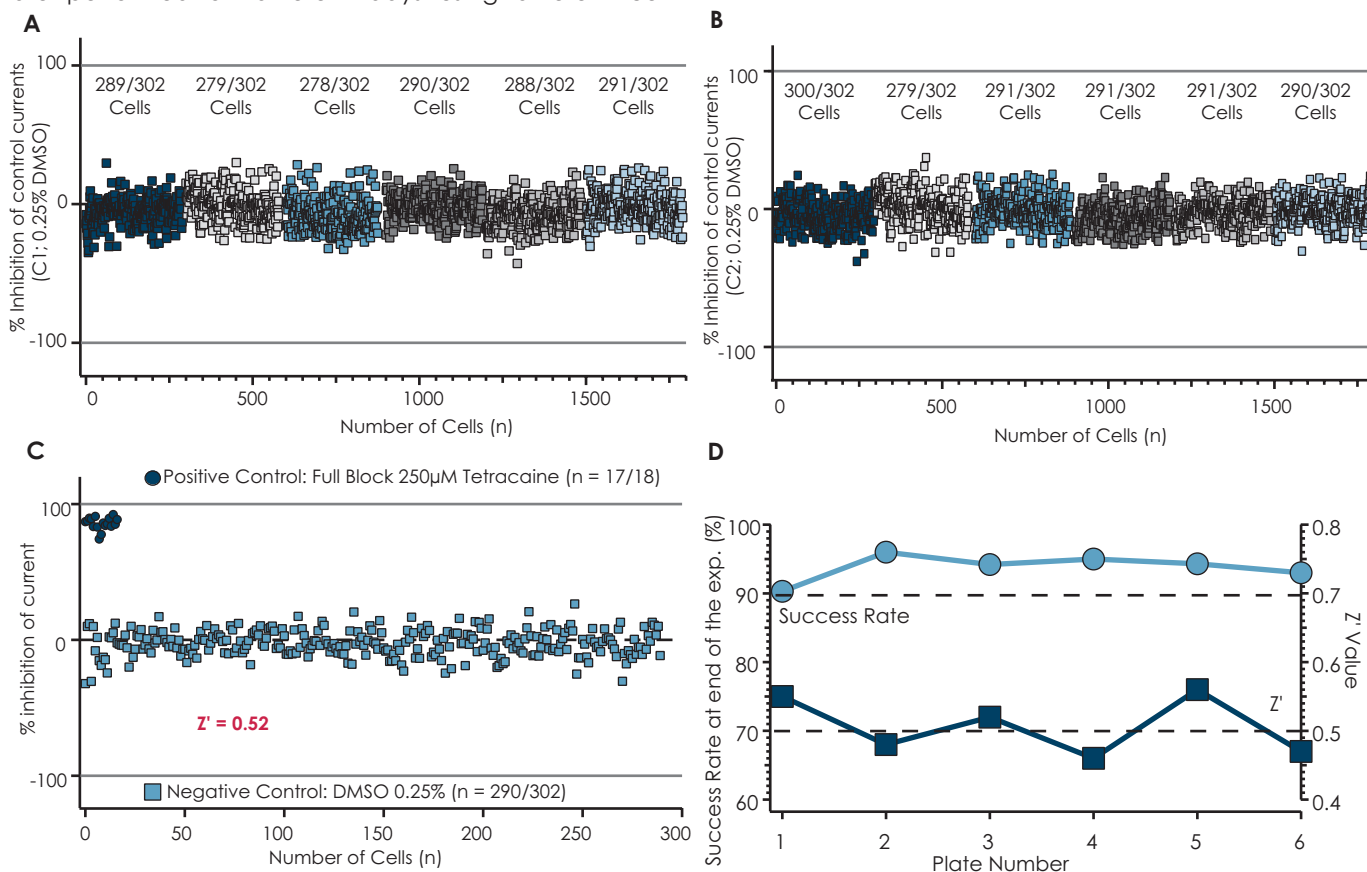


Figure 4: Scatter plots showing % inhibition of current calculated at C1 (A) and C2 (B) after 10 min incubation in 0.25% DMSO using plates on different days using different cell preparations. The stability of the recordings is consistent from day to day. B Calculation of the Z' value for control recordings. Cells were incubated in control solution (0.25% DMSO; light blue squares) or a full block concentration of tetracaine (250 μ M; dark blue circles) for 10 minutes and % inhibition of current at C1 calculated with respect to the start of the experiment. The calculated Z' value > 0.5 reveals this to be an excellent assay for screening hNa_v1.8⁷. D Success rate (light blue circles) at the end of the experiment based on quality control criteria ($I_{peak} > -200$ pA at start; full block with 250 μ M tetracaine; RSeal > 30 M Ω at end) for 6 different plates used on different days. The success rate was consistently $> 90\%$. Also plotted are the Z' values for the same plates.

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In conclusion, hNa_v1.8 expressed in CHO cells can be recorded on the SyncroPatch® 384PE with a high success rate (typically >90% for completed experiments using quality control criteria). The Z-factor, a statistical parameter to evaluate and validate data for HTS⁷, reveals a value typically between 0.5 and 1, confirming that this is an excellent assay for HTS of hNa_v1.8 as the target ion channel. In order to increase current amplitudes and success rates, multi-hole (9X) chips were used so that current amplitudes in the range of -1 to -3 nA and success rates of >90% were routinely achieved. Importantly, there was little spread of normalized current amplitudes and IC₅₀ values across the plate and between plates used on different days using different cell preparations. The SyncroPatch® 384PE is a high throughput and highly reliable automated patch clamp device for recording

hNa_v1.8 and can be used to test compounds for state-dependence. User-friendly software, excellent success rates, multiple additions of compound to each well, availability of single- or multi-hole chips and easy analysis result in high quality, reliable data at a high throughput with an economical cost per data point. Additional optional extras such as temperature control and current clamp increase the scope of experimental potential.

References

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Methods

Cells

CHO cells stably expressing hNa_v1.8 (inducible cell line) were supplied by Charles River (Catalog #: CT6011).

Cell Culture

Cells were cultured and harvested according to Nanion's standard cell culture protocol. Cells were induced to express Na_v1.8 by incubation in tetracycline for at least 24 hours prior to measurements.

Electrophysiology

Whole cell patch clamp recordings were conducted according to Nanion's standard procedure for the SyncroPatch® 384PE using multi-hole chips. Current-voltage recordings were made using voltage steps from -60 mV to 60 mV for 20 ms increasing in 10 mV steps, from a holding potential of -120 mV (leak subtraction protocol used). Pharmacology experiments used two-step protocol to induce slow inactivation.