Toxin pharmacology and MoA data against Na_v1.X channels: Multi-parameter analysis using Sophion Qube high-throughput automated patch clamp platform

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1 ABSTRACT

Voltage-gated sodium (Na_v) channels have been extensively studied as targets for therapeutic indications such as pain, epilepsy and paralysis. However, small molecule interventions have been largely unsuccessful in the clinic, primarily due to challenges in developing compounds with high subtype selectivity to minimise off-target effects.

Many animal venoms have evolved as potent Na_v channel modulators in order to interfere with action potential initiation. Several toxins are highly potent and specific to individual Na_v subtypes, and as such have become widely-used tools in research to elucidate structure and function of the channels. Increasingly, toxins have been used in the development of novel therapeutics as peptide frameworks for new bioactive molecules or targeting scaffolds for drug conjugates.

Ion channels are notoriously challenging to study in drug development, due to long and laborious manual patch clamp approaches. Recent advances in high-throughput automated patch clamp (APC) technologies have allowed high quantities of meaningful data to be generated quickly. Multi-parameter analysis of the data generated can elucidate mechanism of action (MoA), including state-dependence, from a single experiment.

At Charles River, we have previously validated the adaptive voltage protocol on the Sophion Qube 384-well APC platform, allowing Boltzmann fits and $V_{1/2}$ values to be calculated and applied to individual wells, leading to improved precision in generating biophysical and pharmacological data. We have now used these protocols to complete multi-parameter analysis of toxin pharmacology on Na_v1.7 and Na_v1.8 channels.

2 MATERIAL AND METHODS

Cell Culture: CHO T-REX Na_v1.7 and Na_v1.8 were produced at Charles River and are commercially available. Cells were cultured according to their SOP. Na_v1.7 cells were prepared as assay ready vials and thawed into T175 flasks 72 hours prior to experiments. Cells were kept in a serum-free medium on the Qube for up to 4 hours.

Solutions: Extracellular solution (mM): 145 NaCl, 4 KCl, 10 HEPES, 10 Glucose, 1 $MgCl_2$, 2 $CaCl_2$, pH 7.4.

Intracellular solution (mM): 120 CsF, 20 CsCl, 10 NaCl, 10 HEPES, 10 EGTA, pH 7.3.

Compounds: Reference compounds are commercially available. Compound plates were prepared with 0.3% DMSO and 0.01% Pluronic Acid F-127.

Qube Experiments: Experiments were conducted with Sophion Qube software version 2.4.106 (Falcon) using Multi-Hole QChips. The protocol included an Online Boltzmann Fit liquid period, followed by a voltage protocol aimed to test compound effect on resting state and 50% inactivated state (Figure 1). Currents were sampled at 25 kHz with cut off at 5 kHz and Bessel Filtering. All data was generated from a single experiment run for each cell line tested.

Analysis: Data analysis was performed using Qube Analyzer software, GraphPad Prism (8.4) and Dotmatics Vortex (v2020)

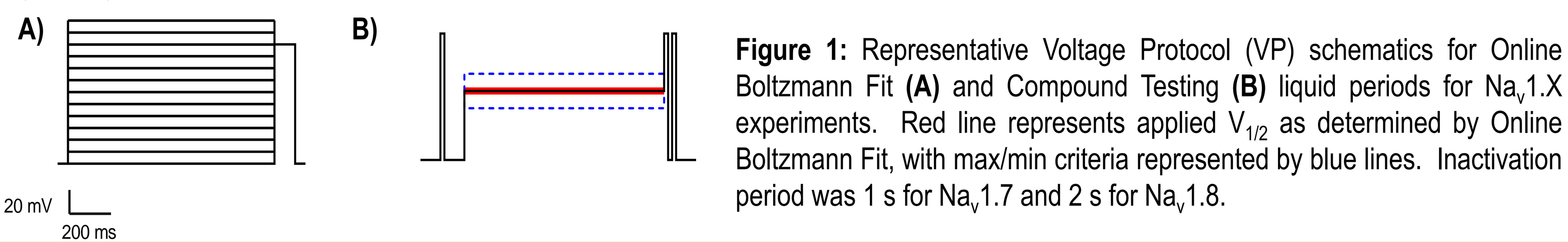


Figure 1: Representative Voltage Protocol (VP) schematics for Online Boltzmann Fit **(A)** and Compound Testing **(B)** liquid periods for Na_v1.X experiments. Red line represents applied $V_{1/2}$ as determined by Online Boltzmann Fit, with max/min criteria represented by blue lines. Inactivation period was 1 s for Na_v1.7 and 2 s for Na_v1.8.

3 RESULTS

3.1 Analysis of QC parameters demonstrate high quality data generation for Na_v1.X channels on Sophion Qube APC platform.

A)

Experiment	Success Rate (%)	Seal Resistance (MΩ)	Cell Capacitance (pF)
Na_v1.7	69	116 ± 2.2	93.8 ± 1.1
Na_v1.8	71	114 ± 1.9	76.9 ± 0.7

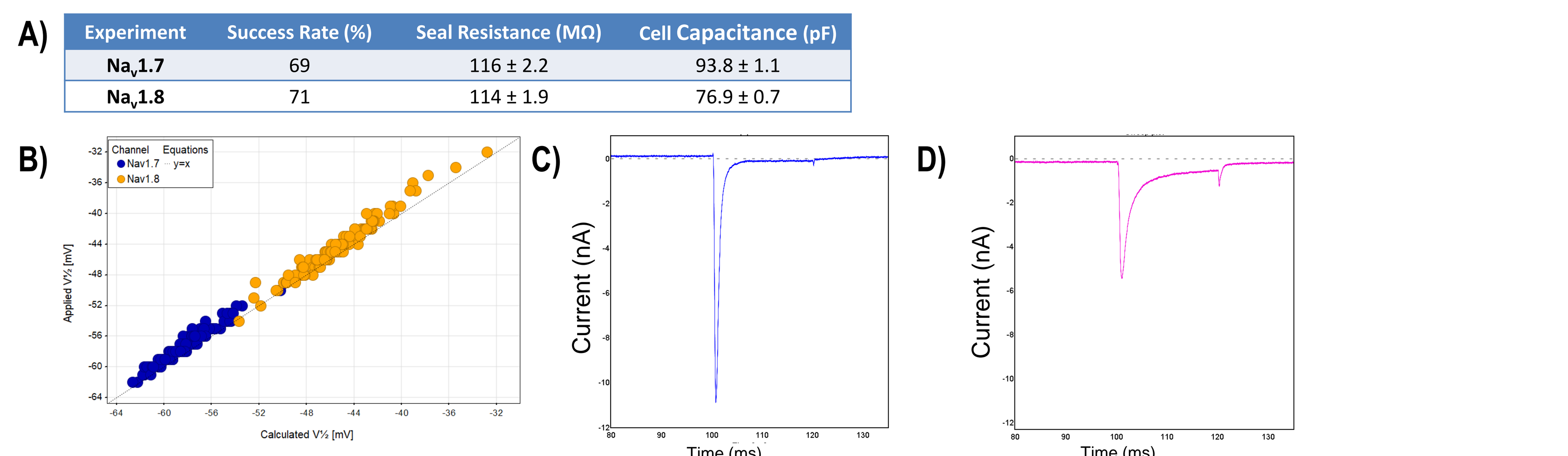
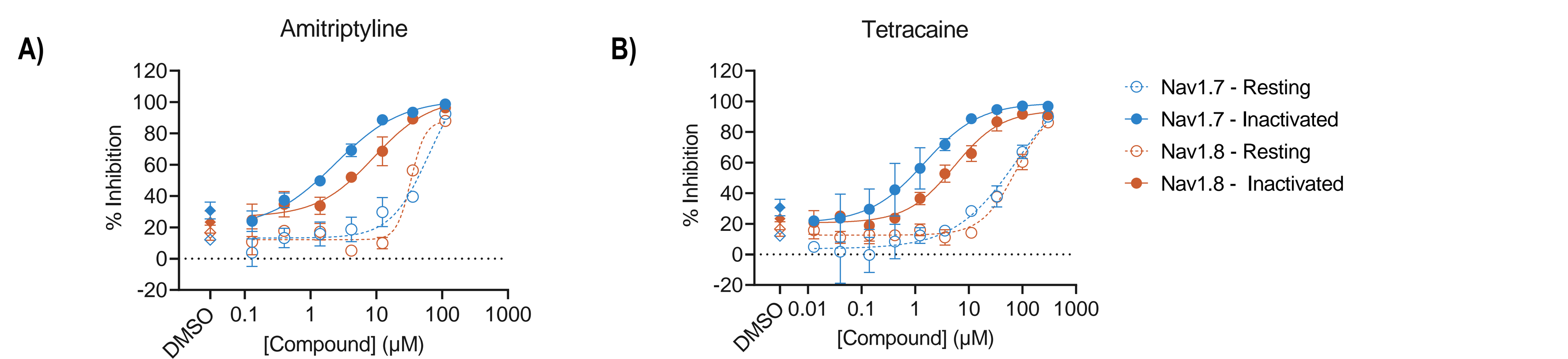


Figure 2: **Na_v1.X assay performance.** **(A)** Table showing assay performance parameters of Na_v1.7 and Na_v1.8 Qube assays. Parameters shown from the 3rd Vehicle Period, following Online Boltzmann Fit. Data shown as Mean ± SEM, n ≥ 265. **(B)** Correlation between $V_{1/2}$ calculated from Boltzmann Plot and $V_{1/2}$ applied by Sophion Qube software during the Online Boltzmann Fit for Na_v1.7 (blue) and Na_v1.8 (orange) assays. Representative current traces are shown for Na_v1.7 **(C)** and Na_v1.8 **(D)** channels.

3.2 Na_v1.X adaptive voltage protocol assay was validated by profiling known small molecule modulators.



C)

Compound	Na_v1.7 IC_{50} (µM)		Na_v1.8 IC_{50} (µM)		Ref.
	Resting	Inactivated	Resting	Inactivated	
Amitriptyline	63.2	2.4 (3.7-8)	33.6	9.2 (8-21.6)	(1, Internal)
Tetracaine	81.2	1.5 (1.5-3)	76.3	5.3 (15-18)	(1, Internal)

Figure 3: Assay validation using reference compounds. Concentration-response curves of amitriptyline **(A)**, and tetracaine **(B)** inhibition of Na_v1.7 (blue) and Na_v1.8 (orange) channels, using both the resting (dotted) and inactivated (solid) state pulses. Data shown as Mean ± SD, n = 2-4. **(C)** Table summarising IC_{50} data (µM) for channel inhibition in line with previously generated values, shown in brackets.

3.3 A selection of toxins with known Na_v1.7 and 1.8 modulating properties were profiled using the adaptive voltage protocol on the Sophion Qube.

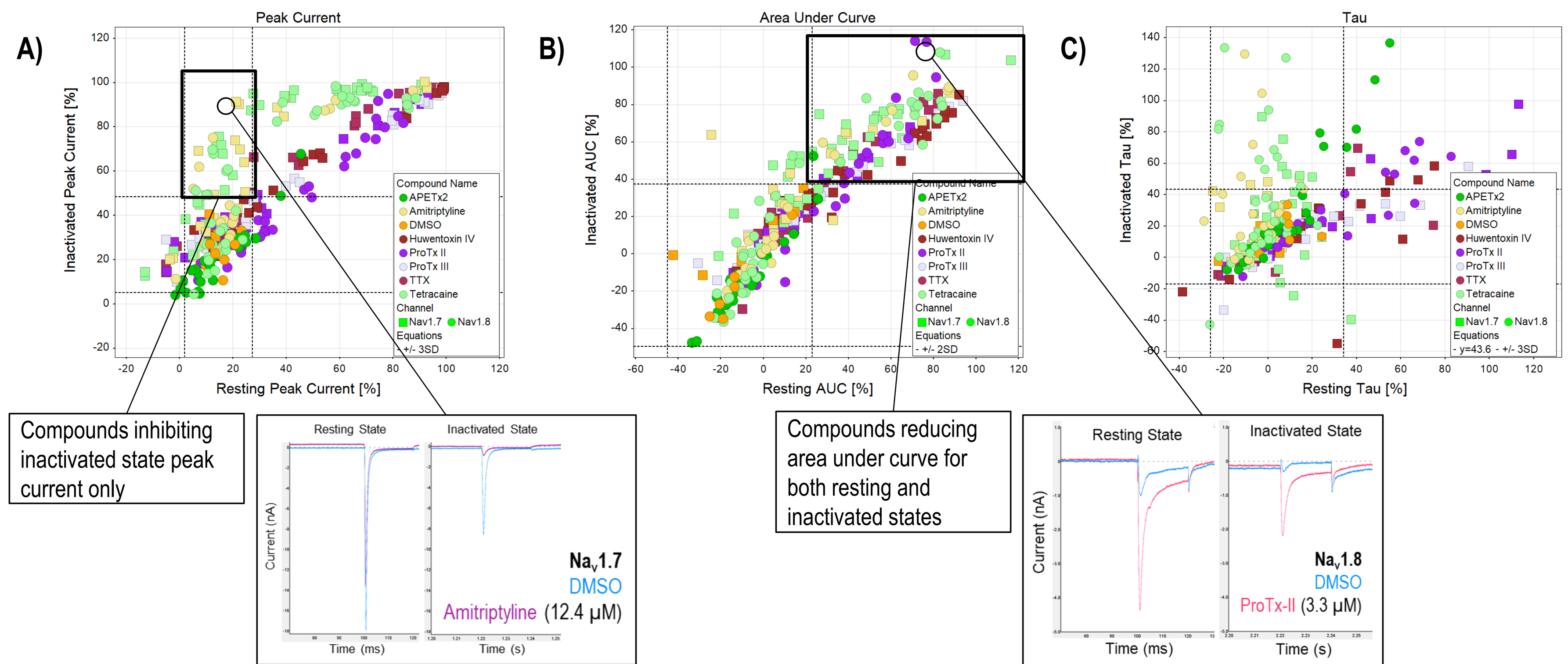


Figure 4: Multi-parameter analysis of toxin pharmacology. Scatter plots comparing compound effect on peak current **(A)**, area under curve **(B)**, and inactivation tau **(C)**, between resting state and 50% inactivated state Na_v1.7 (squares) and Na_v1.8 (circles) channels after application of selected toxins. Toxins were applied as 10 point 1:3 concentration-response curves, top concentration (channel): APETx2 100 µM (1.8); Amitriptyline 100 µM (both); Huwentoxin IV 10 µM (1.7); ProTx II 0.1 µM (1.7), 10 µM (1.8); ProTx III 1 µM (1.7); Tetracaine 100 µM (both); Tetrodotoxin 1 µM (1.7). Peak current and AUC were normalised between vehicle control and full response (0.0). Tau was normalised to vehicle control. Dotted lines and boxes represent hit finding approaches for single point data, with representative current traces.

3.4 Analysis of Sophion Qube data allows inference of toxin mechanism of action from single-well readings.

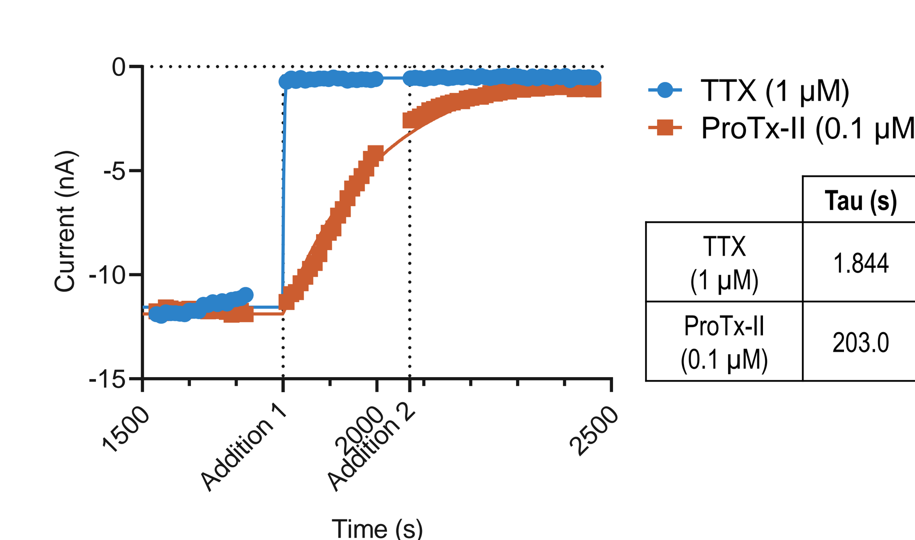
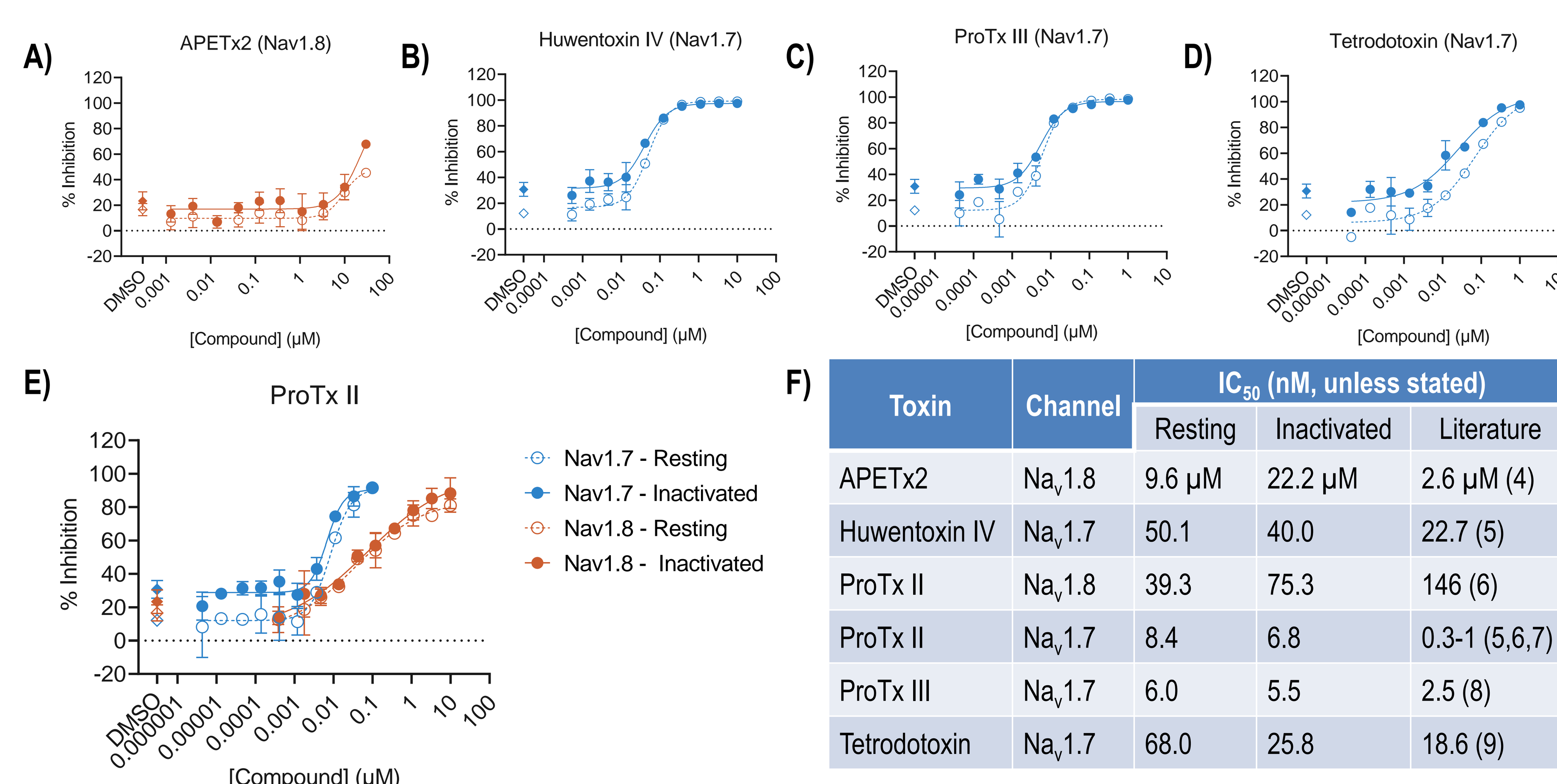


Figure 5: It-plot of resting peak current demonstrating differences in Na_v1.7 channel dynamics following toxin treatment. Both 1 µM TTX (blue) and 0.1 µM ProTx II (orange) result in maximal Na_v1.7 current inhibition, however further information about mechanism of action can be determined from analysis of the It-plot. TTX is known to block the Na_v channel pore [2], resulting in immediate current block, whereas ProTx II interacts with the voltage-sensing domain [3], resulting in a prolonged inhibition. Multi-parameter analysis of Sophion Qube data can allow high quantities of meaningful information about channel modulators to be obtained from a single well reading.

3.5 Pharmacology of toxins was determined, with potency values and selectivity in line with literature.



Toxin	Channel	IC_{50} (nM, unless stated)		
		Resting	Inactivated	Literature
APETx2	Na_v1.8	9.6 µM	22.2 µM	2.6 µM (4)
Huwentoxin IV	Na_v1.7	50.1	40.0	22.7 (5)
ProTx II	Na_v1.8	39.3	75.3	146 (6)
ProTx II	Na_v1.7	8.4	6.8	0.3-1 (5,6,7)
ProTx III	Na_v1.7	6.0	5.5	2.5 (8)
Tetrodotoxin	Na_v1.7	68.0	25.8	18.6 (9)

Figure 6: Potency determination of reference toxins. Concentration-response curves of APETx2 **(A)**, Huwentoxin IV **(B)**, ProTx III **(C)**, Tetrodotoxin **(D)**, and ProTx II **(E)** inhibition of Na_v1.7 (blue) and Na_v1.8 (orange) channels, using both the resting (dotted) and inactivated (solid) state pulses. Data shown as Mean ± SD, n = 2-4. **(F)** Table summarising IC_{50} data (nM, unless stated) for channel inhibition in line with literature values.

4 SUMMARY

The adaptive voltage protocol on the Sophion Qube has been used to successfully generate pharmacology for a range of small molecules and toxins, with potency values in line with the literature. Differences in specific IC_{50} values between this data and published data may be explained by differences in voltage protocols, particularly in the test pulse potential and length of the inactivation period, or use of different cell types. Multi-parameter analysis of Na_v1.X current traces allows mechanism of action data, including state-dependence, to be generated from single-well recordings. Additionally, the expected selectivity of ProTx II was observed, reported to be due to a single amino acid substitution in the voltage sensing domain (3,6). All data presented was obtained from a single Qube experiment for each cell line tested, demonstrating the high quantity and quality of meaningful data which can be obtained using automated patch clamp technology.

References

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