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Characterization of Two Functional Assays and Development of Quality Standards Using Tetrodotoxin and μ-Conotoxin KIIIA as Test Compounds Against Na_V Channels

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Abstract

This study evaluates two commonly used in vitro techniques to assess compound potencies: patch-clamp electrophysiology and fluorescence membrane potential assay. Furthermore, a quality framework to assess Tetrodotoxin and μ-conotoxin KIIIA activity on the voltage-gated sodium (Na_V) receptors Na_V 1.4 and Na_V 1.7 using patch-clamp electrophysiology in a controlled, comparative manner is developed and described. The framework includes verification of cell line receptor overexpression, assay characterization using a well-studied reference compound, data curation, and test compound potency assessment. Data generated here support previous claims that patch-clamp electrophysiology is the gold standard for potency assessments, being a more reproducible and sensitive technique than the fluorescence membrane potential assay. Patch-clamp electrophysiology characterization using nine replicate dose–response curves over three days generated similar half-maximal inhibitory concentration (IC_{50}) values to much larger datasets and the existing literature for the sodium channel cell lines selected for these studies. This framework can be adapted by other laboratories for rapid development and characterization of high-throughput, robust, and efficient functional assays to assess quality and consistency of measurements over time.

Keywords: tetrodotoxin; conotoxin; Na_V channel; patch-clamp electrophysiology; membrane potential assay; characterization; quality metrics

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1. Introduction

Functional assays are critical for evaluating the activity, potency, and safety of novel therapeutic compounds as well as understanding the biological effects of toxins and other chemicals. In functional assays, the ability of a test analyte to modulate the functionality of a biological target is measured. Cell-based functional assays offer a balance between physiological relevance and specific analysis, with experimental or ethical challenges associated with animal work. Despite their widespread use, results from different potency assessments cannot be easily compared in the literature due to differences in instrumentation, experimental design, assay type, and experimental controls.

Two commonly used cell-based functional assays to assess compound potencies are fluorescence membrane potential assays and patch-clamp electrophysiology [1–5]. Fluorescence-based assays, which utilize membrane potential-sensitive dyes to detect changes in ion

flow, are suitable for high-throughput compound screening and assessment. However, because these dyes measure indirect responses to compounds, they often lack the sensitivity required to detect responses [3,5]. Conversely, patch-clamp electrophysiology, which is considered the gold standard for ion channel assessment, allows for the direct and highly sensitive measurement of ion flow across the cell membrane [6,7]. While highly accurate, manual patch-clamp electrophysiology methodologies require greater technical expertise than fluorescence assays and have traditionally been lower throughput [8]. Recent advances in automated, high-throughput patch-clamp electrophysiology alleviate some of these issues, enabling rapid data collection and requiring less technical expertise [7,9,10].

Validation or best-practices guidance is currently limited for functional assays. To date, publications have typically focused on protocols specific to the needs of that study, emphasizing instrument and cell-specific parameters [11–16], but neglecting establishment of general quality standards. The United States Food and Drug Administration (USFDA) published one recommended patch-clamp electrophysiology protocol integrating some best practices from the International Council for Harmonization (ICH) [17–19]. While this protocol did provide guidance on controls, sensitivity, and concentration range criteria, it did not address compound characterization, method characterization, or quality standards, thus highlighting a need for more comprehensive recommendations.

In this study, the inhibitory activity of tetrodotoxin (TTX) and μ-conotoxin KIIIA (KIIIA) on voltage-gated sodium (Na_V) channels Na_V 1.4 and Na_V 1.7 was measured by two different techniques: patch-clamp electrophysiology and membrane potential fluorescence assay. Na_V channels are common targets of both biological toxins and therapeutic drugs due to their key role in regulating action potentials and flow of sodium ions in tissues throughout the body [20–22]. Na_V 1.4 channels are found in skeletal muscles and Na_V 1.7 channels are found in a variety of cells throughout the central and peripheral nervous system. Human exposures to inhibitory toxins can result in symptoms including paralysis, numbness, difficulty breathing, and gastrointestinal distress [23–26].

Rather than focusing on the individual compound potencies, the goal of this study was to improve consistency and comparability between assays by developing more comprehensive quality and performance criteria for functional assays, building on existing guidance from USFDA and ICH. These criteria include validating cell line receptor overexpression, incorporating reference inhibitors to assess assay performance, addressing poor quality data through outlier removal and R^2 criteria, and using a data-driven approach to determine the minimum number of replicates required to generate high-quality data. These criteria were successfully applied to the patch-clamp assays; however, they could not be applied to the fluorescence assays due to highly variable results and the inability to measure KIIIA activity. Overall, this work offers a practical, tested framework for evaluating compounds using patch-clamp electrophysiology, balancing speed and efficiency with rigorous data quality and performance standards.

2. Results

2.1. Verification of Receptor Overexpression

Cell lines overexpressing human Na_V 1.4 and human Na_V 1.7 receptors were procured to assess toxin activity on each receptor type individually. Overexpression of each Na_V channel type was verified by reverse transcription–quantitative polymerase chain reaction (RT-qPCR) prior to functional assay development. The housekeeping gene control beta-actin was expressed in all cell lines tested, but Na_V channel expression was only present in the respective engineered Na_V cell line. The corresponding parental HEK and CHO cell lines did not have detectable levels of mRNA expression for Na_V 1.4 and Na_V 1.7. These

results indicate that toxin functional responses measured with the Na_V cell lines are specific to the engineered receptors (Figure 1).

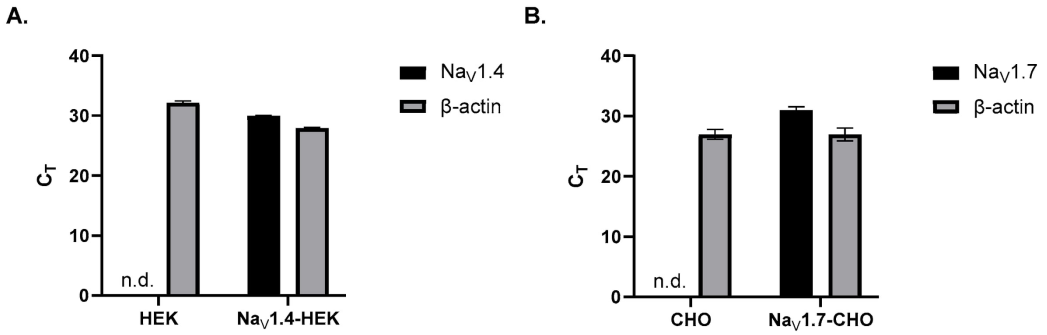

Figure 1. Reverse transcription–quantitative polymerase chain reaction (RT-qPCR) verification of overexpression of voltage-gated sodium (Na_V) channels Na_V 1.4 (**A**) and Na_V 1.7 (**B**) in cell lines was procured for functional assay development, ±standard error of the mean (SEM). Cycle threshold (C_T) value results were collected in triplicate, with b-actin assessed as a constitutively expressed control. n.d. = not detected.

2.2. Patch-Clamp Electrophysiology Method Development and Characterization

After receptor overexpression was verified, patch-clamp electrophysiology protocols were established for Na_V 1.4 and Na_V 1.7 cells to assess potencies of compounds of interest [2,16,27–30]. A reference inhibitor was selected to verify assay performance, characterize assay reproducibility, and serve as a benchmark (or comparative value) with other functional assays. TTX was selected as the reference inhibitor because it is a commonly studied inhibitor of Na_V channels and its potency in terms of half-maximal inhibitory concentrations (IC_{50}) has been well characterized, with reported values ranging from 0.0015 to 0.036 µM [2,16,20–23]. Additionally, parental HEK and CHO cells were assessed as a negative control to verify that the measured responses were specific to the overexpressed Na_V channels.

Vendor recommendations were used to establish the high-throughput patch-clamp electrophysiology methods for each cell line. Following collection of baseline signal and voltage stimulation, TTX produced rapid, reversible inhibition in a concentration-dependent manner (Figure 2). Published IC_{50} values were used to guide the initial concentration range-finding tests for TTX, with a final concentration range of seven TTX solutions from 0.0001 to 1 µM. To characterize the TTX IC50 values for each patch-clamp assay, a minimum of 12 replicate dose–response curves were collected per assay plate, over three independent experimental runs across 3 weeks.

Dose–response curve data were curated by removing curves with two or more outliers, as flagged by default GraphPad Prism outlier removal parameters, or curves with R^2 values less than 0.9. Average TTX IC_{50} and pIC_{50} values were calculated from the remaining dataset curves. Eighty-five percent of the Na_V 1.4 and 96% of the Na_V 1.7 patch-clamp electrophysiology curves collected for TTX passed both the outlier and R^2 criteria, demonstrating the consistency of the established assays. The average TTX IC_{50} was 0.03 µM ($n = 48$) for the Na_V 1.4 assay and 0.019 µM ($n = 44$) for the Na_V 1.7 assay (Table 1). These IC_{50} values were similar to those reported previously (although slightly higher for Na_V 1.4), verifying comparability to other methods previously described and the use of TTX as a reference inhibitor for method characterization. The 95% confidence interval for TTX IC_{50} values can be found in Table 1.

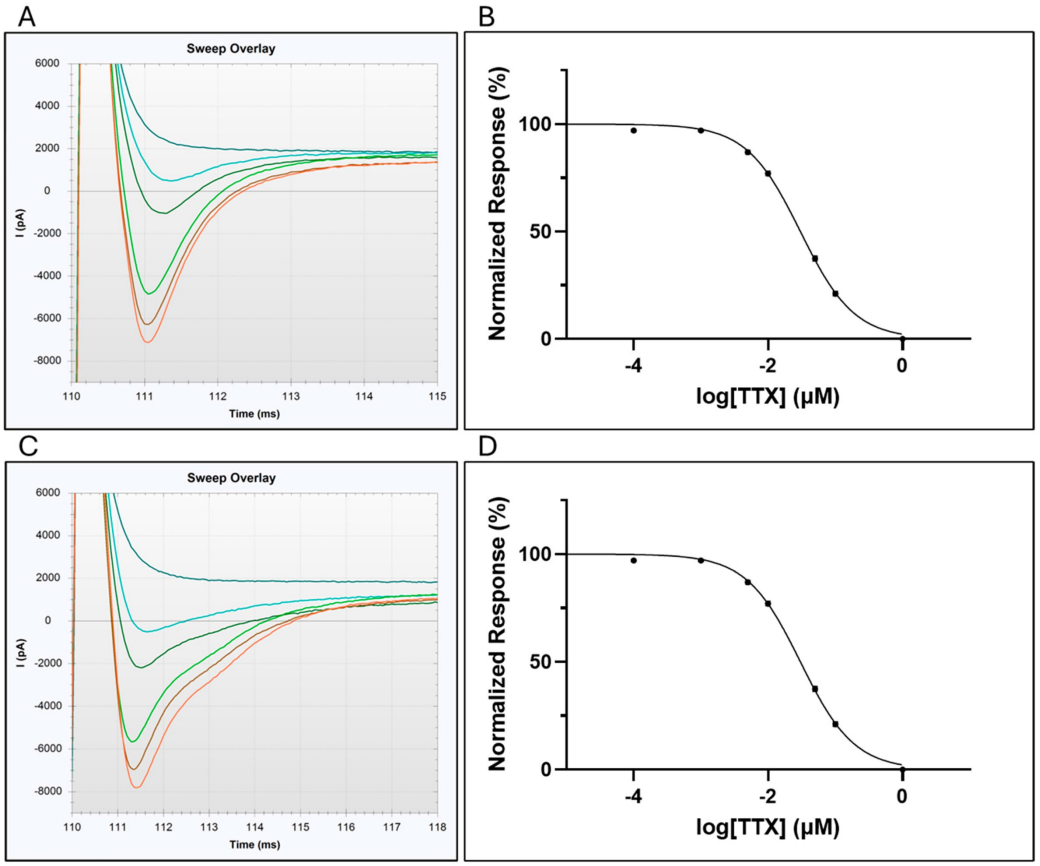

Figure 2. Tetrodotoxin (TTX) signal sweeps and dose–response curves for Na_V 1.4 ((**A**) and (**B**), respectively) and Na_V 1.7 ((**C**) and (**D**), respectively). Dose–response curve data points represent averages from Na_V 1.4 (n = 48) and Na_V 1.7 (n = 44) ± SEM. Colored traces represent different concentrations of TTX. Color assignments were automatically set by the instrument software.

Table 1. Average TTX responses from Na_V 1.4 (n = 48) and Na_V 1.7 (n = 44) dose–response curves collected by patch-clamp electrophysiology over three days. IC50: half-maximal inhibitory concentration; CV: coefficient of variation; CI: confidence interval.

Receptor	pIC_{50} (μM)	%CV for pIC_{50}	IC_{50} (μM)	IC_{50} 95% CI (μM)
Na_V 1.4	−1.52	11.2%	0.030	0.027 to 0.034
Na_V 1.7	−1.72	10.3%	0.019	0.017 to 0.022

2.3. Assay Statistical and Replicate Analysis

A large number of replicate dose–response curves (48 for Na_V 1.4 and 44 for Na_V 1.7) were collected in this study for the reference compound TTX to assess the performance of the developed patch-clamp electrophysiology protocols (Table 1). However, the low coefficient of variation % (CV) (~10%) of the TTX response characterized using both Na_V 1.4 and Na_V 1.7 patch-clamp electrophysiology protocols indicated that fewer replicates may be sufficient. To test this hypothesis, all replicate curves from each run were compared to smaller subset replicate datasets from the same run to determine the minimum number of replicates required to obtain a statistically equivalent pIC_{50} value. A three-replicate subset collected over three runs (n = 9 total) was sufficient to obtain a pIC_{50} that was not statistically different from the pIC_{50} of the dataset comprising all replicate curves. Because no significant differences were observed between these complete and minimal (n = 9) datasets, 3σ (standard deviation) confidence limits were established as pass/fail criteria using the minimal dataset pIC_{50} values to assess future assay performance. We found

that 94% of the Na_V 1.4 and 86% of the Na_V 1.7 replicate pIC_{50} values generated from the dose–response curves were within the minimal dataset 3σ confidence limits (Figure 3). This indicates it would be unlikely for multiple dose–response curves to fail on a given day without a larger underlying issue. The replicate pIC_{50} values that fell outside of the passing limits were left in Figure 3 to provide a visualization of the failure rate. We used the first three replicate dose–response curves from each run to generate the minimal 3σ confidence limits reported in Table 2 and found that choosing different replicates did not affect results.

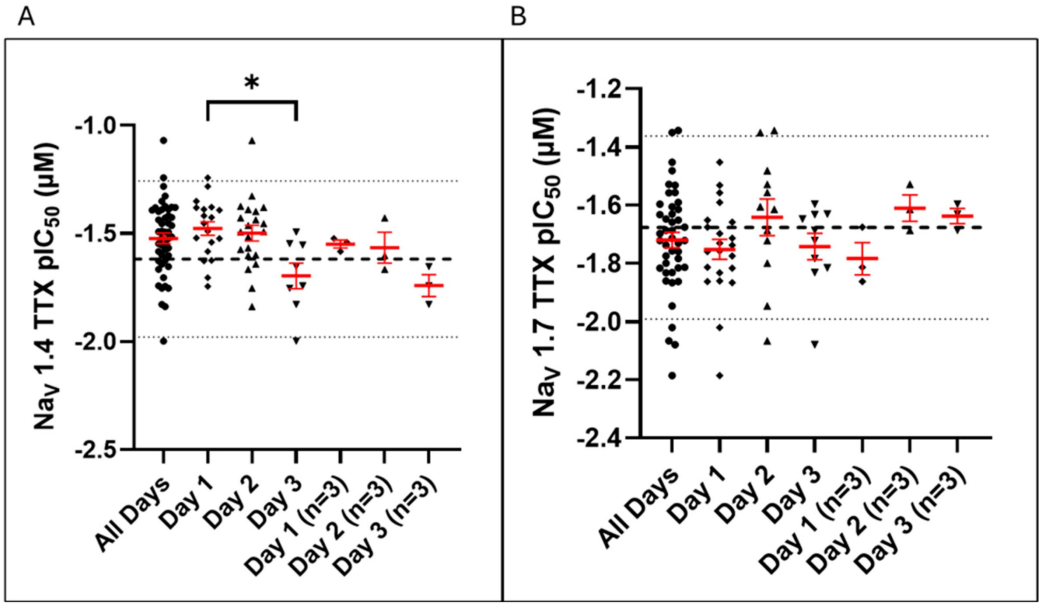

Figure 3. Comparison of Na_V 1.4 (**A**) and Na_V 1.7 (**B**) TTX patch-clamp electrophysiology pIC_{50} values from complete and subset dose–response datasets. The bold horizontal dashed line represents the average pIC_{50} value calculated from the first three dose–response curves collected daily over three days ($n = 9$). The two horizontal dotted lines represent the 3σ confidence limits associated with the minimal dataset. The mean and error bars represent the pIC_{50} value and standard error for each pool of results. All means were compared pairwise by analysis of variance (ANOVA), with the single significant comparison result marked by a bracket and an asterisk.

Table 2. Average TTX pIC_{50}, IC_{50}, and 3σ confidence limits from Na_V 1.4 and Na_V 1.7 ($n = 9$) dose–response curves collected by patch-clamp electrophysiology over three days.

Receptor	Average pIC_{50} (μM)	pIC_{50} 3σ Confidence Limits (μM)	Average IC_{50} (μM)
Na_V 1.4	−1.618	−1.979 to −1.257	0.024
Na_V 1.7	−1.677	−1.991 to −1.363	0.021

The average TTX pIC_{50} values for the minimal datasets were −1.618 μM for the Na_V 1.4 cell line and −1.677 μM for the Na_V 1.7 cell line (Table 2). Further analysis showed that triplicate pIC_{50} values from a single run did not differ significantly from measurements pooled over two or three days (Figure 4). This suggests that similar compounds with unknown potencies could be screened with high measurement confidence using much smaller replicate sets on a single day, as long as the reference compound pIC_{50} falls within the established 3σ confidence limits, verifying assay performance.

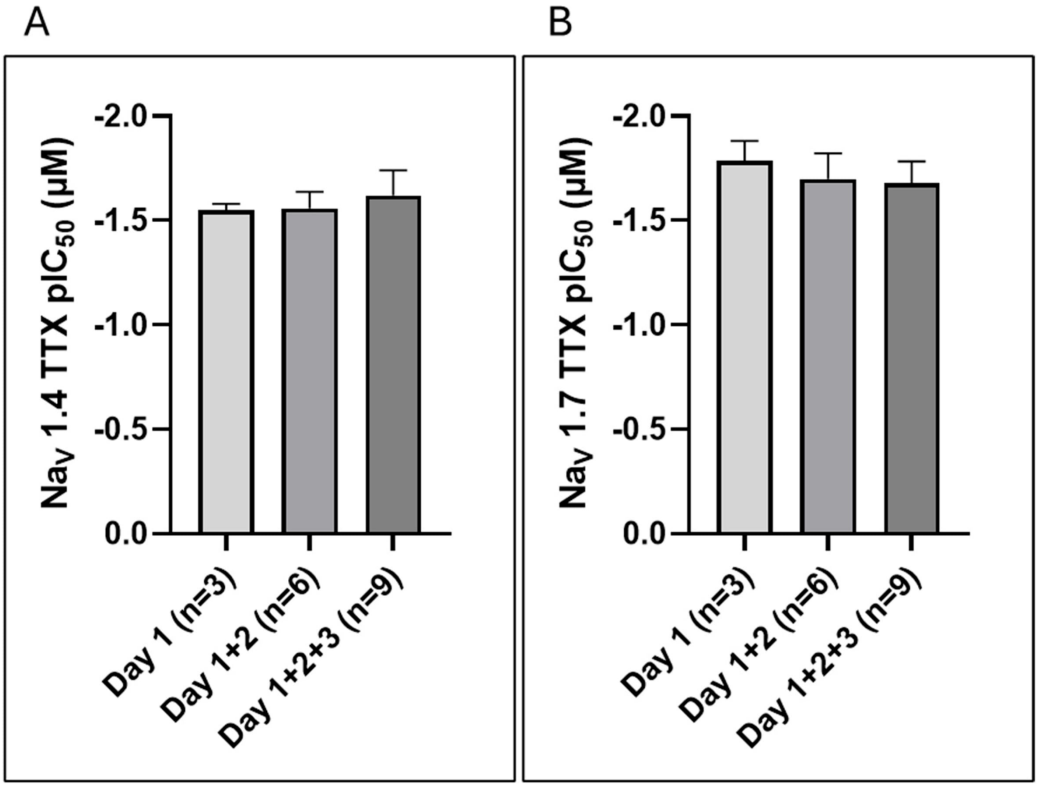

Figure 4. Average TTX pIC_{50} values for Na_V 1.4 (**A**) and Na_V 1.7 (**B**) cell lines. Each bar graph measures the average of pooled measurements ± standard deviation over three days. All means were compared by ANOVA with no significant differences observed between days.

2.4. KIIIA Potency Assessment by Patch-Clamp Electrophysiology

μ-Conotoxin KIIIA was selected as a test compound to assess the validity of the method and proposed workflow for potency screening. It is of interest both as a therapeutic and toxic compound, and has known site 1 activity on both Na_V 1.4 and Na_V 1.7 receptors [31–35]. For each run with a minimum of three passing replicate TTX dose–response curves (to verify assay performance against the characterized limits), a minimum of three replicate KIIIA dose–response curves were assessed for potency. To verify the hypothesis that a single experimental run was sufficient to generate an accurate potency measurement for a test compound, data was collected over three days and evaluated. Na_V 1.4 and Na_V 1.7 cells were exposed to sequential KIIIA concentrations ranging from 0.01 to 50 μM after voltage stimulation. The experimental pattern layout for each replicate curve followed the same format as was used for TTX experiments. KIIIA inhibited both Na_V 1.4 and Na_V 1.7 cell lines in a dose-dependent manner (Figure 5). The IC_{50} for KIIIA was 0.612 μM ($n = 3$) for the Na_V 1.4 cells and 1.65 μM ($n = 3$) for the Na_V 1.7 cells (Table 3). While greater variability in KIIIA IC_{50} values was observed compared to TTX, the average pIC_{50} values measured over three days were not significantly different (Figure 6). The KIIIA IC_{50} values measured were consistent with previously published results (0.065–5.39 μM) [28,34,36,37]. This test case indicates that triplicate potency measurements on a single day may be sufficient to generate high-confidence IC_{50} values for test compound screening purposes when characterized reference compounds are included in each experiment to verify assay performance.

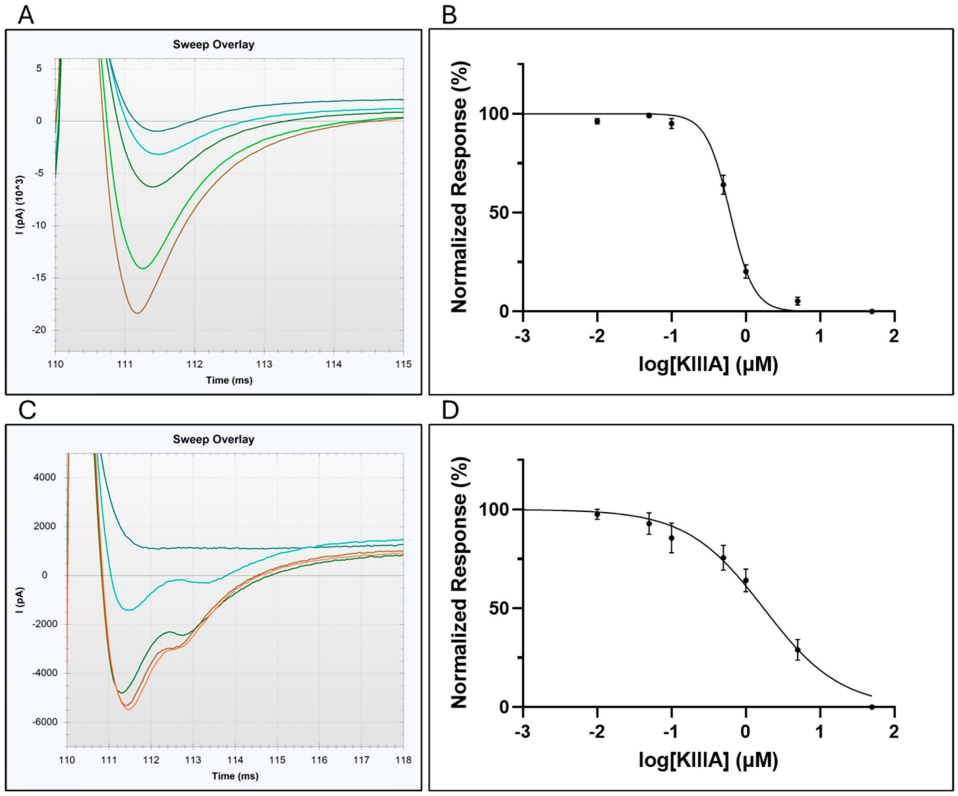

Figure 5. KIIIA signal sweeps and dose–response curves for Na_V 1.4 ((**A**) and (**B**), respectively) and Na_V 1.7 ((**C**) and (**D**), respectively). Dose–response curve data points represent averages from Na_V 1.4 ($n = 3$) and Na_V 1.7 ($n = 3$) ± SEM. Colored traces represent different concentrations of KIIIA. Color assignments were automatically set by the instrument software.

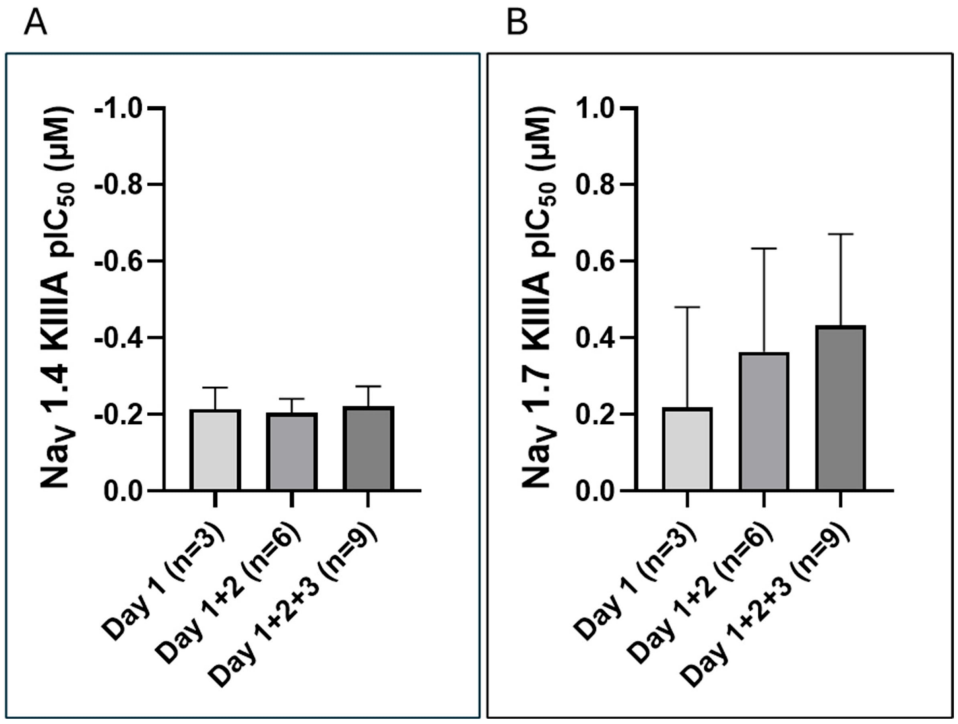

Figure 6. Average KIIIA pIC_{50} values for Na_V 1.4 (**A**) and Na_V 1.7 (**B**) cell lines. Each bar graph measures the average of pooled measurements ± standard deviation over three days. All means were compared by ANOVA with no significant differences observed between days.

Table 3. Average KIIIA pIC_{50} values and IC_{50} values ($n = 3$).

Receptor	Average pIC_{50} (μM)	Standard Deviation of pIC_{50} (μM)	Average IC_{50} (μM)
Na_V 1.4	−0.214	0.057	0.612
Na_V 1.7	0.219	0.262	1.65

2.5. Fluorescence Assay Development and Characterization

To determine if this approach could be applied to other types of potency assays and for direct IC_{50} comparison with patch-clamp electrophysiology, a fluorescence membrane potential dye assay was developed to characterize TTX responses on both Na_V 1.4 and Na_V 1.7 cell lines. In each assay, the Na_V cell line was stimulated with a 100 μM final concentration of the agonist veratridine to generate a fluorescence response. Each experimental replicate column also contained a well with no inhibitor to act as a vehicle control and signal baseline. Parental HEK and CHO cells, lacking Na_V channel expression, were unresponsive to veratridine addition, confirming that assay response is specific to the Na_V overexpressing cell lines. To test receptor inhibition, cells were incubated with varying concentrations of TTX prior to the addition of veratridine to generate dose–response curves. TTX concentrations ranged from 0.0005 μM to 5 μM. TTX dose–response curves were generated for each cell line, including data from at least three independent experiments over three or more weeks to characterize method performance. TTX inhibited fluorescence responses in both Na_V cell lines in a concentration-dependent manner (Figure 7). However, only 8/39 (21%) of the Na_V 1.4 and 23/44 (52%) of the Na_V 1.7 TTX dose–response curves met the quality standards of ≤1 outlier/curve and $R^2 \geq 0.9$, previously established and applied to patch-clamp electrophysiology. Fluorescence assay pIC_{50} values were within the range of previously published values and similar to patch-clamp electrophysiology results [30]. The utility of this fluorescence assay is limited by both the high percentage of failed curves and the greater curve-to-curve variability. Compared to the patch-clamp electrophysiology results, a prohibitively large number of replicate curves would be required to generate similarly high-quality results (Table 4). Despite the reduced robustness of the assay compared to patch-clamp electrophysiology, KIIIA was still tested using the assay. Although concentrations up to 5 μM KIIIA were tested, no inhibitory activity was detected (Figure 7).

Table 4. Average TTX pIC_{50}, IC_{50}, and %CV results from Na_V 1.4 ($n = 8$) and Na_V 1.7 ($n = 23$) dose–response curves collected by fluorescence assay over three days.

Receptor	pIC_{50} (μM)	%CV for pIC_{50}	IC_{50} (μM)	IC_{50} 95% CI (μM)
Na_V 1.4	−1.311	15.5%	0.049	0.033 to 0.072
Na_V 1.7	−2.218	19.5%	0.0061	0.0039 to 0.0093

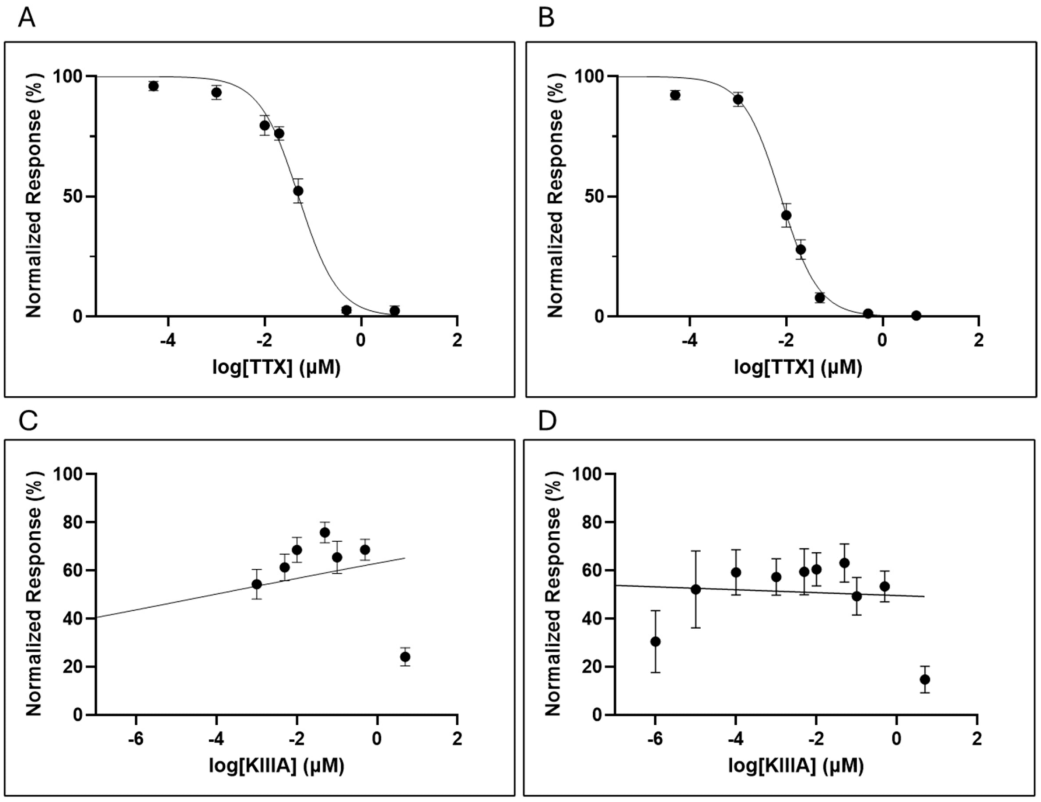

Figure 7. Dose–response curves showing TTX and KIIIA inhibition of Na_V 1.4 ((**A**,**C**), respectively) and Na_V 1.7 ((**B**,**D**), respectively) receptors by fluorescence assay. TTX dose–response curve data points (**A**,**B**) represent averages from $n = 8$ (Na_V 1.4) and $n = 23$ (Na_V 1.7) replicates ± SEM. KIIIA dose–response curve data points (**C**,**D**) represent averages from $n = 27$ (Na_V 1.4) and $n = 21$ (Na_V 1.7) replicates ± SEM. Sigmoidal curves could not be generated for KIIIA due to a lack of concentration-dependent inhibition of Na_V 1.4 or Na_V 1.7 receptors.

3. Discussion

Automated patch-clamp electrophysiology and fluorescence assays were developed to assess Na_V channel inhibition by conotoxins, using KIIIA as a test compound and TTX as the reference inhibitor. Due to limited method validation guidance for these types of assays, a quality characterization workflow was established. The workflow described here integrates components from previous best practices and guidance and builds on that groundwork to create a more comprehensive best practices framework that can be more widely applied [17–19]. Previous best practices incorporated into our workflow include use of sufficient inhibitor concentrations spanning a wide inhibitory range, sufficient cells, vehicle and negative controls, full inhibition samples, and suitable seal resistance. In addition to these parameters, verification of cell line receptor overexpression, application of outlier and R^2 criteria to dose–response curves as quality control parameters, incorporation of a reference inhibitor dose–response curve as an assay performance control, and use of commercially available materials where possible were incorporated to improve consistency, comparability, and confidence in results. In addition, to increase efficiency, the minimum number of replicate dose–response curves required to obtain equivalent results to much larger datasets was determined. This framework can be applied to other compounds, receptors, and instrumentation to develop similar high-quality assessments of potency.

While consistent potency measurements were made for all compounds and cell lines assessed by patch-clamp electrophysiology, similar measurements could not be generated by fluorescence assay for KIIIA using either Na_V 1.4 or Na_V 1.7 cells. Similar fluorescence assay observations have been reported by other groups and were attributed to various

sensitivity [3,38], compound [39,40], and cell line-specific issues [5]. Another potential assay limitation is that fluorescence membrane potential assays use an indirect fluorescence measurement to detect pooled response across cells in the entire well, instead of reporting direct changes to ion flow of individual cells. Although KIIIA functional responses were not detected by the fluorescence assay in our system, TTX responses were measured and were consistent with the literature and the potency values obtained by patch-clamp electrophysiology. This indicates the assay was functioning properly and that the inability to measure responses from KIIIA is likely not due to failures of instrumentation, kit reagents, cell lines, or KIIIA materials.

While the quality framework established here can be adapted to other test systems, replicate requirements and assay reproducibility should be verified when assessing other cell lines, instrumentation, and compounds of interest. Overall curve failure rate based on these criteria can be included in the determination of how many replicates to utilize, balancing throughput with the risk of having too few passing curves. Removing outliers and curves with low R^2 values significantly improved the coefficient of variation and confidence intervals associated with each IC_{50} but had a minimal impact on the average IC_{50} values generated in these datasets. Incorporating these quality parameters, especially with smaller numbers of replicate curves, can provide additional levels of confidence in reported IC_{50} values. However, the impact of these parameters on other toxin and receptor combinations should be assessed to verify these findings.

There are several limitations associated with this study. First, the long-term reproducibility of TTX IC_{50} results in our test system is unknown, as our experiments were conducted over a relatively short 2–3-month span. It is possible that over longer periods of time, measured IC_{50} values may drift outside the initially characterized confidence limits and need to be recharacterized due to factors such as use of different material lots. Strategies such as incorporating toxicity equivalency factors (TEFs), which report test compound IC_{50} values as a ratio relative to a reference compound, like TTX, may help improve comparability of results across experiments where raw IC_{50} values do not precisely align and recharacterization is unfeasible [41–45]. It is also possible that other receptor and agonist combinations may require different numbers of replicates than reported here, which would need to be assessed on an individual basis. The general replicate workflow described here could be applied to unique receptor and agonist combinations to determine minimal replicate requirements and modified, as needed, to meet individual laboratory quality requirements.

4. Materials and Methods

4.1. Materials

HEK293 parental cells, CHO-K1 parental cells, and FBS were purchased from ATCC (Manassas, VA, USA). HEK293 cells overexpressing human Na_V 1.4 were received as a generous gift from Pacific Northwest National Laboratory (PNNL) (Richland, WA, USA). Tissue culture flasks, Non-Essential Amino Acids Solution (NEAA), Trypan Blue, 96-well plates, and CHO Serum-Free Media were purchased from Corning (Corning, NY, USA). DMEM/F-12 GlutaMAX™ and Ham's/F12 media were purchased from Gibco (Gaithersburg, MD, USA). Penicillin–Streptomycin Solution (Pen/Strep), Blasticidin, HBSS, Accutase, HEK Serum Free Media, HEPES, Veratridine, DMSO, NaCl, KCl, $CaCl_2$ dihydrate, Glucose, $MgCl_2$, TEA-Cl, NaOH, Sucrose, CsF, CsOH, and EGTA were purchased from MilliporeSigma (Burlington, MA, USA). Puromycin was purchased from AG Scientific (San Diego, CA, USA). CHO cells overexpressing human Na_V 1.7 were purchased from Charles River Laboratories (Burlington, MA, USA). Tetracycline was purchased from Zymo Research (Irvine, CA, USA). TaqMan Gene Expression Cells-to-C_T Kit and Zeocin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). FLIPR Membrane Potential Assay

Kits were purchased from Molecular Devices, LLC (San Jose, CA, USA). Tetrodotoxin and μ-conotoxin KIIIA were purchased from Alomone Labs (Jerusalem, Israel).

4.2. Buffer Formulations

4.2.1. Inhibitory Compound Preparation

TTX and KIIIA were purchased as tubes containing 1 mg of lyophilized toxin. Tubes were diluted in water to a concentration of 1 mg/mL, distributed into 100 μL aliquots, and stored at −20 °C until ready for use.

4.2.2. Membrane Potential Fluorescence Assay

Media for fluorescence assays comprised serum-free media supplemented with 25 mM HEPES (SFM + HEPES). FLIPR Dye Solution was prepared according to assay kit protocol instructions. A 37.1 mM stock solution of veratridine in DMSO was diluted to a 500 μM working solution in FLIPR Assay Buffer.

4.2.3. Patch-Clamp Electrophysiology

Media for patch-clamp electrophysiology comprised serum-free media supplemented with 25 mM HEPES (SFM + HEPES). Cell loading buffer consisted of 145 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$ dihydrate, 10 mM HEPES, 10 mM glucose, 1 mM $MgCl_2$ hexahydrate, and 10 mM TEA-Cl, adjusted to pH 7.4 with NaOH and 305 mmol/kg with sucrose. Intracellular solution consisted of 135 mM CsF, 10 mM HEPES, 5 mM CsOH, 1 mM EGTA, and 10 mM NaCl, adjusted to pH 7.2 with CsOH and 295 mmol/kg with sucrose. Extracellular solution consisted of 145 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$ dihydrate, 10 mM HEPES, 10 mM glucose, 1 mM $MgCl_2$ hexahydrate, and 10 mM TEA-Cl, adjusted to pH 7.4 with NaOH and 305 mmol/kg osmolarity with sucrose. These solutions follow vendor recommendations for optimal performance.

4.3. Cell Culture

Parental HEK and CHO cell lines were passaged according to ATCC product documentation. Na_V 1.4 HEK cells were passaged according to ATCC product documentation for HEK cell lines, modified for selection antibiotic specifications provided by PNNL. Na_V 1.7 CHO cells were passaged according to Charles River Laboratories product documentation. Accutase was used in place of trypsin for all cell passaging to maintain optimal cell surface receptor expression.

Human embryonic kidney parental cells and stably overexpressing Na_V 1.4-HEK293 (Na_V 1.4) cells were grown in T-75 or T-150 flasks with DMEM/F-12 GlutaMAX™ media supplemented with 10% FBS, 100X NEAA, and 50X pen/strep at 37 °C, 5% CO_2, and 95% humidity under sterile conditions. Na_V 1.4 cells were further supplemented with 10 μg/mL of puromycin as a selection antibiotic, per instructions received from PNNL. Chinese hamster ovary parental cells and tetracycline-inducible cells overexpressing the Na_V 1.7 receptor were grown in T-75 or T-150 flasks with Ham's/F12 media supplemented with 10% FBS, 100X NEAA, and 50X pen/strep at 37 °C, 5% CO_2, and 95% humidity under sterile conditions. Na_V 1.7 cells were further supplemented with 10 μg/mL blasticidin and 400 μg/mL zeocin as selection antibiotics. A 3 μg/mL final concentration of tetracycline was added to fresh media of Na_V 1.7 cells 24 h prior to experiments. Cells were passaged at 70–80% confluency. HBSS was used to wash cells during passaging and experiments, and Accutase was used to resuspend cells. A Countess 2 Automated Cell Counter (Thermo Fisher Scientific, Carlsbad, CA, USA) and trypan blue were used to count cells and determine live/dead percentages.

Chinese hamster ovary cells were passaged 3 times per week, seeding either 3×10^6 or 5×10^6 cells per T-150 flask. Na_V 1.7 cells were received from Charles River Laboratories

at passage number 37, and all experiments were conducted prior to passage number 75. Control Chinese hamster ovary cells did not have a passage number associated with their ATCC CoA, and all experiments were conducted within 36 passages of receipt.

Human embryonic kidney cells were passaged 2 times per week, seeding either 3×10^6 or 4×10^6 cells per T-150 flask. Na_V 1.4 cells were received from PNNL labeled as passage number 10, and all experiments were conducted prior to passage number 35. Control human embryonic kidney cells were received from ATCC at passage number 37, and all experiments were conducted prior to passage number 62.

4.4. RT-qPCR

All PCR experiments were conducted according to assay kit and instrument documentation. After cell number optimization experiments were completed, two-step RT-PCR was conducted on Na_V 1.4, Na_V 1.7, parental CHO, and parental HEK cells using TaqMan Gene Expression Assay Kits for Na_V 1.4-encoding gene *SCN4A*, Na_V 1.7-encoding gene *SCN9A*, and β-actin-encoding gene *ACTB* using the protocol in the TaqMan Gene Expression Cells-to-C_T Kit (Thermo Fisher Scientific, Waltham, MA, USA). All samples were run on a QuantStudio 7 Pro RT-qPCR machine (Thermo Fisher Scientific, Waltham, MA, USA) and results were analyzed and plotted in GraphPad Prism software (Boston, MA, USA).

SCN4A gene expression assay kit was Thermo Fisher Scientific Assay ID Hs01109480_m1, corresponding to sequence accession number NM_000334.4, with an amplicon length of 70, starting at base 4100. *SCN9A* gene expression assay kit was Thermo Fisher Scientific Assay ID Hs01076699_m1, corresponding to sequence accession number NM_002977.3, with an amplicon length of 69, starting at base 1946. *ACTB* gene expression assay kit was Thermo Fisher Scientific Assay ID Hs01060665_g1, corresponding to sequence accession number NM_001101.3, with an amplicon length of 63, starting at base 208.

4.5. Fluorescence Membrane Potential Assay

4.5.1. Assay Plate Setup

Final concentrations of agonist and inhibitors were selected following range-finding experiments based on vendor recommendations and expected values. The agonist, veratridine, was added to a final concentration of 100 μM, which produced a strong but non-saturating signal that was able to be inhibited by TTX in a concentration-dependent manner. Inhibitory compound dilutions were prepared from frozen aliquots prepared in 4.2.1 using a Tecan Fluent 1080 (Tecan, Mannedorf, Switzerland). Inhibitory compound final concentrations were, in μM, for TTX: 0, 0.0005, 0.001, 0.01, 0.02, 0.05, 0.5, and 5; and for KIIIA: 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 5.

Fluorescence studies were conducted using a Molecular Devices Flexstation 3 (Molecular Devices, LLC, San Jose, CA, USA) instrument and FLIPR Membrane Potential Assay Kit, following manufacturer-recommended instrument parameters and protocol, as follows. There was 1 × 96-well plate per experiment of Na_V 1.4 HEK, control HEK, Na_V 1.7 CHO, or control CHO cells grown to 85–90% confluency at the center 1 mm^2 of each well and prepared for the assay. Cell culture media was replaced with 75 μL per well of SFM + HEPES. After incubating at 37 °C for 30 min, 100 μL per well of FLIPR Dye Solution was added. After incubating at 37 °C for another 30 min, the 96-well plate was loaded into the instrument along with a 96-well plate containing agonist and inhibitor, and the assay was started.

4.5.2. Instrument Conditions

The Flexstation 3 instrument conducted all transfers of agonist and inhibitor into the cell plate during the assay run. The assay proceeded in a column-wise manner, starting with inhibitor addition to each well in a column. During the assay, 25 μL of inhibitor was

dispensed into each well of the plate containing cells. Following a 5 min incubation at room temperature, 50 µL of veratridine working solution was dispensed into each well of the plate containing cells. Fluorescence signal was read at 550 nm for each well, starting 5 min after inhibitor addition. Agonist was added 17 s into the fluorescence reading period, which comprised 23 total reads over 90 s. The peak signal was recorded and subtracted from the average baseline recorded prior to compound addition.

4.6. Patch-Clamp Electrophysiology

4.6.1. Assay Plate Setup

The whole-cell patch-clamp electrophysiology studies were conducted using an Ionflux Mercury HT (Cell Microsystems, Durham, NC, USA), with vendor-recommended parameters as follows. There were 3 × T-150 cell culture flasks per experiment; Na_V 1.4 HEK, control HEK, Na_V 1.7 CHO, or control CHO cells were grown to 75–85% confluency and harvested, followed by centrifugation at 200× *g* for 1 min. The pellet was resuspended in 15 mL per T-150 flask of SFM + HEPES, transferred to a T-25 flask, and mixed at 90 rpm for 30–60 min. The cells were then counted, centrifuged at 200× *g* for 1 min, and resuspended in cell loading buffer. Following a final count, the concentration was adjusted to 8×10^6–1×10^7 cells/mL, with at least 2.5 mL final volume required for an experiment. Cells and buffer solutions were dispensed onto the patch-clamp electrophysiology plate according to manufacturer instructions. Inhibitory compound dilutions were prepared in extracellular solution from frozen aliquots prepared in Section 4.2.1, and all reagents were dispensed into the assay plate using a Tecan Fluent 1080. Inhibitory compound final concentrations were, in µM, for TTX—0, 0.0001, 0.001, 0.005, 0.01, 0.05, 0.1, and 1—and for KIIIA—0, 0.01, 0.05, 0.1, 0.5, 1, 5, and 50. Final concentrations were selected following range-finding experiments based on vendor recommendations and expected values from previous publications.

4.6.2. Instrument Conditions

The patch-clamp system completed a prime phase (120 s), a cell trapping phase (135 s), a break (100 s), and a data acquisition phase (1120 s) using manufacturer-recommended parameters. Peak signal was recorded and subtracted from the average baseline recorded prior to compound addition. The voltage stimulation period was 2 s. This period comprised 1800 ms of holding at −80 mV, followed by pulses to −100 mV for 10 ms, −120 mV for 100 ms, −10 mV for 30 ms, and −100 mV for 60 ms. The sample rate was 20 kHz.

4.7. Statistical Analysis

Statistical analysis was conducted using GraphPad Prism version 10.5.0. For PCR, cycle threshold (C_T) values were analyzed, and a linear regression was calculated and plotted.

For both FLIPR and patch-clamp electrophysiology, the raw data was exported to Microsoft Excel. The data was then transferred to Prism, normalized, and analyzed by individual curve with a nonlinear curve fit. Outliers were identified and removed, followed by removal of non-passing curves, using default Prism outlier removal parameters. A curve was considered non-passing if it contained more than 1 outlier point or had an R^2 value of less than 0.9. pIC_{50} values from passing curves were then pooled, and outlier pIC_{50} values were identified and removed, using default Prism outlier removal parameters. The mean, standard deviation, and 95% confidence intervals of these pooled pIC_{50} values were calculated [46–49].

To establish characterization limits for $n = 3$ curves per day, the first three passing pIC_{50} values from each experiment were pooled; mean, standard deviation, and 95% confidence intervals were calculated. A one-way ANOVA was used to determine if any day's pIC_{50} values or $n = 3$ pIC_{50} values differed significantly, using default Prism parameters, all

pairwise comparisons, the Holm–Šídák multiple comparisons correction, and an alpha of 0.05.

4.8. Waste Handling and Disposal

All potentially toxic, hazardous, and biohazardous materials were stored, handled, and disposed of in accordance with established CDC risk assessments, procedures, biosafety, and chemical safety guidelines.

5. Conclusions

In conclusion, the potencies of two commonly studied inhibitors of Na_V channels (TTX and KIIIA) were assessed by both patch-clamp electrophysiology and fluorescence membrane potential assay. Each assay was set up following instrument and assay manufacturer recommendations and existing best practices. Additional quality standards were devised and implemented to further verify assay consistency and improve comparability between functional assays. These quality standards included verification of cell line receptor overexpression, assay characterization using a well-studied reference compound, data curation to remove poor-quality data, and collection of sufficient replicates to minimize result bias. While patch-clamp electrophysiology results consistently corroborated previously published IC_{50} results for both toxins, the fluorescence assay was inconsistent and unable to measure the potency of KIIIA. The proposed quality framework described here can be applied by others in the development of high-throughput, robust, and efficient functional assays for analysis of uncharacterized toxins or compounds of interest to improve assay consistency and result comparability.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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Abbreviations

The following abbreviations are used in this manuscript:

USFDA	United States Food and Drug Administration
ICH	International Council for Harmonization
TTX	Tetrodotoxin
Na_V	Voltage-Gated Sodium Channel
RT-qPCR	Reverse Transcription–Quantitative Polymerase Chain Reaction
HEK	Human Embryonic Kidney
CHO	Chinese Hamster Ovary
SEM	Standard Error of the Mean
IC_{50}	Half-Maximal Inhibitory Concentration
CV	Coefficient of Variation
CI	Confidence Interval
σ	Standard Deviation
ANOVA	Analysis Of Variance
FLIPR	Fluorescent Imaging Plate Reader
PNNL	Pacific Northwest National Laboratory
C_T	Cycle Threshold
TEF	Toxicity Equivalency Factor

References

1. Alvarez Baron, C.; Zhao, J.; Yu, H.; Ren, M.; Thiebaud, N.; Guo, D.; Vegesna, G.; Hsiao, C.-H.; DePalma, R.; Mistry, S.; et al. Multi-laboratory comparisons of manual patch clamp hERG data generated using standardized protocols and following ICH S7B Q&A 2.1 best practices. *Sci. Rep.* **2025**, *15*, 29995. [CrossRef] [PubMed]
2. Campàs, M.; Reverté, J.; Tudó, À.; Alkassar, M.; Diogène, J.; Sureda, F.X. Automated Patch Clamp for the Detection of Tetrodotoxin in Pufferfish Samples. *Mar. Drugs* **2024**, *22*, 176. [CrossRef] [PubMed]
3. Elkins, R.C.; Davies, M.R.; Brough, S.J.; Gavaghan, D.J.; Cui, Y.; Abi-Gerges, N.; Mirams, G.R. Variability in high-throughput ion-channel screening data and consequences for cardiac safety assessment. *J. Pharmacol. Toxicol. Methods* **2013**, *68*, 112–122. [CrossRef] [PubMed]
4. Inserra, M.C.; Kompella, S.N.; Vetter, I.; Brust, A.; Daly, N.L.; Cuny, H.; Craik, D.J.; Alewood, P.F.; Adams, D.J.; Lewis, R.J. Isolation and characterization of α-conotoxin LsIA with potent activity at nicotinic acetylcholine receptors. *Biochem. Pharmacol.* **2013**, *86*, 791–799. [CrossRef] [PubMed]
5. Liu, K.; Samuel, M.; Tillett, J.; Hennan, J.K.; Mekonnen, B.; Soloveva, V.; Harrison, R.K.; Paslay, J.W.; Larocque, J. High-throughput screening for Kv1.3 channel blockers using an improved FLIPR-based membrane-potential assay. *J. Biomol. Screen* **2010**, *15*, 185–195. [CrossRef] [PubMed]
6. Hill, C.L.; Stephens, G.J. An Introduction to Patch Clamp Recording. *Methods Mol. Biol.* **2021**, *2188*, 1–19. [CrossRef] [PubMed]
7. Priest, B.T.; Cerne, R.; Krambis, M.J.; Schmalhofer, M.A.; Wakulchik, M.; Wilenkin, B.; Burris, K.D. Automated Electrophysiology Assays. In *Assay Guidance Manual*; Markossian, S., Grossman, A., Baskir, H., Arkin, M., Auld, D., Austin, C., Baell, J., Brimacombe, K., Chung, T.D.Y., Coussens, N.P., et al., Eds.; Eli Lilly & Company and the National Center for Advancing Translational Sciences: Bethesda, MD, USA, 2004.
8. Milligan, C.J.; Möller, C. Automated planar patch-clamp. *Methods Mol. Biol.* **2013**, *998*, 171–187. [CrossRef] [PubMed]
9. Obergrussberger, A.; Rinke-Weiß, I.; Goetze, T.A.; Rapedius, M.; Brinkwirth, N.; Becker, N.; Rotordam, M.G.; Hutchison, L.; Madau, P.; Pau, D.; et al. The suitability of high throughput automated patch clamp for physiological applications. *J. Physiol.* **2022**, *600*, 277–297. [CrossRef] [PubMed]
10. Seibertz, F.; Rapedius, M.; Fakuade, F.E.; Tomsits, P.; Liutkute, A.; Cyganek, L.; Becker, N.; Majumder, R.; Clauß, S.; Fertig, N.; et al. A modern automated patch-clamp approach for high throughput electrophysiology recordings in native cardiomyocytes. *Commun. Biol.* **2022**, *5*, 969. [CrossRef] [PubMed]
11. Toh, M.F.; Brooks, J.M.; Strassmaier, T.; Haedo, R.J.; Puryear, C.B.; Roth, B.L.; Ouk, K.; Pin, S.S. Application of High-Throughput Automated Patch-Clamp Electrophysiology to Study Voltage-Gated Ion Channel Function in Primary Cortical Cultures. *SLAS Discov.* **2020**, *25*, 447–457. [CrossRef] [PubMed]
12. He, B.; Soderlund, D.M. Human embryonic kidney (HEK293) cells express endogenous voltage-gated sodium currents and Na v 1.7 sodium channels. *Neurosci. Lett.* **2010**, *469*, 268–272. [CrossRef] [PubMed]
13. BenchChem Technical Support Team. Application Notes and Protocols for the Experimental Study of Sodium Ion Channels. 2026. Available online: https://pdf.benchchem.com/46/Application_Notes_and_Protocols_for_the_Experimental_Study_of_Sodium_Ion_Channels.pdf (accessed on 15 May 2026).

14. Ole Paulsen Lab. Whole-Cell Patch-Clamp Recordings for Characterizing Neuronal Electrical Properties. Available online: https://docs.axolbio.com/wp-content/uploads/patch-clamp-protocol-final.pdf (accessed on 15 May 2026).
15. Liu, Y. Electrophysiological Studies of Voltage-Gated Sodium Channels Using QPatch HT, an Automated Patch-Clamp System. *Curr. Protoc. Pharmacol.* **2014**, *65*, 11.14.1–11.14.45. [CrossRef] [PubMed]
16. Matthews, R.M.; Bradley, E.; Griffin, C.S.; Lim, X.R.; Mullins, N.D.; Hollywood, M.A.; Lundy, F.T.; McGarvey, L.P.; Sergeant, G.P.; Thornbury, K.D. Functional expression of Na(V)1.7 channels in freshly dispersed mouse bronchial smooth muscle cells. *Am. J. Physiol. Cell Physiol.* **2022**, *323*, C749–C762. [CrossRef] [PubMed]
17. USFDA. *Recommended Voltage Protocols to Study Drug-Cardiac Ion Channel Interactions Using Recombinant Cell Lines*; Food and Drug Administration: Silver Spring, MD, USA, 2021.
18. Harmonization of ICF. ICH E14/S7B Implementation Working Group: Clinical and Nonclinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential Questions and Answers 2020. Available online: https://database.ich.org/sites/default/files/ICH_E14-S7B_QAs_Step2_2020_0827_0.pdf (accessed on 15 May 2026).
19. Harmonization of ICF. The Non-Clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals S7B 2005. Available online: https://database.ich.org/sites/default/files/S7B_Guideline.pdf (accessed on 15 May 2026).
20. Bennett, D.L.; Clark, A.J.; Huang, J.; Waxman, S.G.; Dib-Hajj, S.D. The Role of Voltage-Gated Sodium Channels in Pain Signaling. *Physiol. Rev.* **2019**, *99*, 1079–1151. [CrossRef] [PubMed]
21. Kwong, K.; Carr, M.J. Voltage-gated sodium channels. *Curr. Opin. Pharmacol.* **2015**, *22*, 131–139. [CrossRef] [PubMed]
22. Noreng, S.; Li, T.; Payandeh, J. Structural Pharmacology of Voltage-Gated Sodium Channels. *J. Mol. Biol.* **2021**, *433*, 166967. [CrossRef] [PubMed]
23. How, C.K.; Chern, C.-H.; Huang, Y.-C.; Wang, L.-M.; Lee, C.-H. Tetrodotoxin poisoning. *Am. J. Emerg. Med.* **2003**, *21*, 51–54. [CrossRef] [PubMed]
24. Isbister, G.K.; Kiernan, M.C. Neurotoxic marine poisoning. *Lancet Neurol.* **2005**, *4*, 219–228. [CrossRef] [PubMed]
25. Kapil, S.; Hendriksen, S.; Cooper, J.S. Cone Snail Toxicity. In *StatPearls 2023*; StatPearls Publishing: Treasure Island, FL, USA, 2023.
26. Bulaj, G.; West, P.J.; Garrett, J.E.; Watkins, M.; Zhang, M.-M.; Norton, R.S.; Smith, B.J.; Yoshikami, D.; Olivera, B.M. Novel conotoxins from Conus striatus and Conus kinoshitai selectively block TTX-resistant sodium channels. *Biochemistry* **2005**, *44*, 7259–7265. [CrossRef] [PubMed]
27. Katikou, P.; Gokbulut, C.; Kosker, A.R.; Campàs, M.; Ozogul, F. An Updated Review of Tetrodotoxin and Its Peculiarities. *Mar. Drugs* **2022**, *20*, 47. [CrossRef] [PubMed]
28. French, R.J.; Yoshikami, D.; Sheets, M.F.; Olivera, B.M. The tetrodotoxin receptor of voltage-gated sodium channels–perspectives from interactions with micro-conotoxins. *Mar. Drugs* **2010**, *8*, 2153–2161. [CrossRef] [PubMed]
29. Lim, X.R.; Bradley, E.; Griffin, C.S.; Hollywood, M.A.; Sergeant, G.P.; Thornbury, K.D. Fast voltage-dependent sodium (Na(V)) currents are functionally expressed in mouse corpus cavernosum smooth muscle cells. *Br. J. Pharmacol.* **2022**, *179*, 1082–1101. [CrossRef] [PubMed]
30. Vickery, R.G.; Amagasu, S.M.; Chang, R.; Mai, N.; Kaufman, E.; Martin, J.; Hembrador, J.; O'Keefe, M.D.; Gee, C.; Marquess, D.; et al. Comparison of the pharmacological properties of rat Na(V)1.8 with rat Na(V)1.2a and human Na(V)1.5 voltage-gated sodium channel subtypes using a membrane potential sensitive dye and FLIPR. *Recept. Channels* **2004**, *10*, 11–23. [PubMed]
31. Kimball, I.H.; Nguyen, P.T.; Olivera, B.M.; Sack, J.T.; Yarov-Yarovoy, V. Molecular determinants of μ-conotoxin KIIIA interaction with the human voltage-gated sodium channel Na(V)1.7. *Front. Pharmacol.* **2023**, *14*, 1156855. [CrossRef] [PubMed]
32. Pan, T.; Li, X.; Zhou, Y.; Wang, N.; Song, X.; Zhang, Z.; Jiang, T.; Huang, Z.; Wang, W.; Wu, G.; et al. Design, Synthesis, and Structure–Activity Relationship Study of μ-Conotoxin KIIIA toward NaV1.4. *J. Med. Chem.* **2026**, *69*, 7856–7868. [CrossRef] [PubMed]
33. Van Der Haegen, A.; Peigneur, S.; Tytgat, J. Importance of position 8 in μ-conotoxin KIIIA for voltage-gated sodium channel selectivity. *Febs J.* **2011**, *278*, 3408–3418. [CrossRef] [PubMed]
34. Zhang, M.M.; Green, B.R.; Catlin, P.; Fiedler, B.; Azam, L.; Chadwick, A.; Terlau, H.; McArthur, J.R.; French, R.J.; Gulyas, J.; et al. Structure/function characterization of micro-conotoxin KIIIA, an analgesic, nearly irreversible blocker of mammalian neuronal sodium channels. *J. Biol. Chem.* **2007**, *282*, 30699–30706. [CrossRef] [PubMed]
35. Zhao, Z.; Pan, T.; Chen, S.; Harvey, P.J.; Zhang, J.; Li, X.; Yang, M.; Huang, L.; Wang, S.; Craik, D.J.; et al. Design, synthesis, and mechanism of action of novel μ-conotoxin KIIIA analogues for inhibition of the voltage-gated sodium channel Nav1.7. *J. Biol. Chem.* **2023**, *299*, 103068. [CrossRef] [PubMed]
36. McArthur, J.R.; Singh, G.; McMaster, D.; Winkfein, R.; Tieleman, D.P.; French, R.J. Interactions of key charged residues contributing to selective block of neuronal sodium channels by μ-conotoxin KIIIA. *Mol. Pharmacol.* **2011**, *80*, 573–584. [CrossRef] [PubMed]
37. Tran, H.N.T.; McMahon, K.L.; Deuis, J.R.; Vetter, I.; Schroeder, C.I. Structural and functional insights into the inhibition of human voltage-gated sodium channels by μ-conotoxin KIIIA disulfide isomers. *J. Biol. Chem.* **2022**, *298*, 101728. [CrossRef] [PubMed]

38. Menegon, A.; Pitassi, S.; Mazzocchi, N.; Redaelli, L.; Rizzetto, R.; Rolland, J.F.; Poli, C.; Imberti, M.; Lanati, A.; Grohovaz, F. A new electro-optical approach for conductance measurement: An assay for the study of drugs acting on ligand-gated ion channels. *Sci. Rep.* **2017**, *7*, 44843. [CrossRef] [PubMed]
39. Zhao, F.; Li, X.; Jin, L.; Zhang, F.; Inoue, M.; Yu, B.; Cao, Z. Development of a Rapid Throughput Assay for Identification of hNav1.7 Antagonist Using Unique Efficacious Sodium Channel Agonist, Antillatoxin. *Mar. Drugs* **2016**, *14*, 36. [CrossRef] [PubMed]
40. Zheng, W.; Spencer, R.H.; Kiss, L. High throughput assay technologies for ion channel drug discovery. *Assay. Drug Dev. Technol.* **2004**, *2*, 543–552. [CrossRef] [PubMed]
41. World Health Organization. *FAO/WHO Technical Paper on Toxicity Equivalence Factors for Marine Biotoxins Associated with Bivalve Molluscs*; World Health Organization: Rome, Italy, 2016.
42. Botana, L.M.; Hess, P.; Munday, R.; Nathalie, A.; DeGrasse, S.L.; Feeley, M.; Suzuki, T.; Berg, M.v.D.; Fattori, V.; Gamarro, E.G.; et al. Derivation of toxicity equivalency factors for marine biotoxins associated with Bivalve Molluscs. *Trends Food Sci. Technol.* **2017**, *59*, 15–24. [CrossRef]
43. Aballay-González, A.; Gallardo-Rodriguez, J.J.; Silva-Higuera, M.; Rivera, A.; Ulloa, V.; Delgado-Rivera, L.; Rivera-Belmar, A.; Astuya, A. Neuro-2a cell-based assay for toxicity equivalency factor-proposal and evaluation in Chilean contaminated shellfish samples. *Food Addit. Contam. Part A* **2020**, *37*, 162–173. [CrossRef] [PubMed]
44. Alkassar, M.; Sanchez-Henao, A.; Reverté, J.; Barreiro, L.; Rambla-Alegre, M.; Leonardo, S.; Mandalakis, M.; Peristeraki, P.; Diogène, J.; Campàs, M. Evaluation of Toxicity Equivalency Factors of Tetrodotoxin Analogues with a Neuro-2a Cell-Based Assay and Application to Puffer Fish from Greece. *Mar. Drugs* **2023**, *21*, 432. [CrossRef] [PubMed]
45. Reverté, J.; Sanchez-Henao, A.; Reverté, J.; Barreiro, L.; Rambla-Alegre, M.; Leonardo, S.; Mandalakis, M.; Peristeraki, P.; Diogène, J.; Campàs, M. Toxicity Equivalency Factors for Tetrodotoxin Analogues Determined with Automated Patch Clamp on Voltage-Gated Sodium Channels in Neuro-2a Cells. *J. Agric. Food Chem.* **2024**, *72*, 18192–18200. [CrossRef] [PubMed]
46. Srinivasan, B.; Lloyd, M.D. Dose-Response Curves and the Determination of IC(50) and EC(50) Values. *J. Med. Chem.* **2024**, *67*, 17931–17934. [PubMed]
47. Srinivasan, B.M.D. Lloyd, Quantitation and Error Measurements in Dose-Response Curves. *J. Med. Chem.* **2025**, *68*, 2052–2056. [CrossRef] [PubMed]
48. Paolini, G.V.; Lyons, R.A.; Laflin, P. How desirable are your IC50s? A way to enhance screening-based decision making. *J. Biomol. Screen* **2010**, *15*, 1183–1193. [CrossRef] [PubMed]
49. Carpenter, J.R. A method for presenting and comparing dose-response curves. *J. Pharmacol. Methods* **1986**, *15*, 283–303. [CrossRef] [PubMed]

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