A functional Nav1.7-NavAb chimera with a reconstituted high affinity ProTx-II binding site

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ProTx-II binding to functional Na\textsubscript{v}1.7- Na\textsubscript{v}Ab chimera

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Abbreviations:
Na\textsubscript{v} : Voltage Gated Sodium Channel
CIP : Congenital Insensitivity to Pain
STS : Surrogate Target Strategy
VSD2 : Voltage Sensor Domain 2
SPR : Surface Plasmon Resonance
SPA : Scintillation Proximity Assay
IEM : Inherited Erythromelalgia
PEPD : Paroxysmal Extreme Pain Disorder
CIP : Congenital Insensitivity to Pain
Ca\textsubscript{v} : Voltage-Gated Calcium Channel
MOL #108712

Abstract

NaV1.7 is implicated in human pain perception by genetics. Rare gain of function mutations in NaV1.7 lead to spontaneous pain in humans whereas loss of function mutations result in congenital insensitivity to pain (CIP). Hence, agents that specifically modulate the function of NaV1.7 have the potential to yield novel therapeutics to treat pain. The complexity of the channel and the challenges to generate recombinant cell lines with high NaV1.7 expression have led to a surrogate target strategy (STS) approach employing chimeras with the bacterial channel, NaVAb. In this report we describe the design, synthesis, purification and characterization of a chimera containing part of the voltage sensor domain 2 (VSD2) of NaV1.7. Importantly, this chimera, DII S1-S4, forms functional sodium channels and is potently inhibited by the NaV1.7 VSD2 targeted peptide toxin ProTx-II. Further, we show by [125I]ProTx-II binding and Surface Plasmon Resonance (SPR) that the purified DII S1-S4 protein retains high affinity ProTx-II binding in detergent. We employed the purified DII S1-S4 protein to create a Scintillation Proximity Assay (SPA) suitable for high throughput screening. The creation of a NaV1.7- NaVAb chimera with the VSD2 toxin binding site provides an important tool for the identification of novel NaV1.7 inhibitors and for structural studies to understand the toxin-channel interaction.
Introduction

The voltage-gated sodium channel Na\textsubscript{v}1.7, encoded by the gene SCN9A, has a compelling genetic link to human pain perception (Dib-Hajj et al., 2013). Rare mutations in SCN9A which result in overactive Na\textsubscript{v}1.7 channels cause autosomal dominant pain disorders such as inherited erythromelalgia (IEM) and paroxysmal extreme pain disorder (PEPD) (reviewed in (Dib-Hajj et al., 2010). In contrast, rare mutations which result in loss of function of Na\textsubscript{v}1.7 lead to the recessively inherited congenital insensitivity to pain (CIP) (Cox et al., 2006; Cox et al., 2010; Goldberg et al., 2007). Reduced pain-related behaviors have also been observed in mice with global knock out of Na\textsubscript{v}1.7 or mice with selective knock out in specific sensory and/or sympathetic neurons (Gingras et al., 2014; Minett et al., 2014; Minett et al., 2012). These results are consistent with an important role for Na\textsubscript{v}1.7 in pain perception and suggest that selective inhibitors of Na\textsubscript{v}1.7 may have broad therapeutic potential in a variety of pain conditions.

Na\textsubscript{v}1.7, like all eukaryotic voltage-gated Na\textsubscript{v} channels, is comprised of four domains linked by cytoplasmic loops (Catterall, 2012). Each domain possesses a voltage sensor and pore module. Based on numerous studies over the last 60 years, Na\textsubscript{v} channels are expected to have a central pore formed from the S5-S6 segments and four voltage sensing domains (VSD1-VSD4) each comprised of the S1-S4 segments. Structural studies of bacterial Na\textsubscript{v} channels (Payandeh et al., 2011; Zhang et al., 2012) and the voltage-gated Ca\textsubscript{v} channel Ca\textsubscript{v}1.1 (Wu et al., 2016; Wu et al., 2015) and the cockroach NavPaS channel (Shen et al., 2017) support this general arrangement. The modular architecture has facilitated pharmacological and structural studies of chimeric channels generated by the transfer of VSDs into related channels. (Ahuja et al., 2015; Alabi et al., 2007; Bosmans et al., 2008; Klint et al., 2015).
The discovery of Nav1.7 inhibitors with sufficient selectivity across the Nav 1.X family to be effective pain therapeutic agents has proven to be a significant challenge. However, the identification of selective, small molecule aryl sulfonamide Nav1.7 inhibitors targeting the voltage sensor domain 4 (VSD4) have reinvigorated these efforts (Focken et al., 2016; McCormack et al., 2013). Furthermore, landmark structural studies of a chimeric channel where a portion of the VSD4 is grafted onto the bacterial Nav channel, NavAb, has provided key molecular insight into the determinants of selectivity (Ahuja et al., 2015). Despite the considerable promise of selective small molecule inhibitors targeting NaV1.7 VSD4, confirmation of clinical efficacy has not yet been reported for these agents (Cao et al., 2016; Jones et al., 2016).

In addition to VSD4, several other regions of the Nav1.7 channel may be useful for targeting, including the outer pore (Thomas-Tran and Du Bois, 2016; Walker et al., 2012), inner vestibule (Bagneris et al., 2014), and the other VSDs. Notably, VSD2 is the site of interaction of the selective peptide inhibitor ProTx-II (Schmalhofer et al., 2008) and related peptides (Klint et al., 2015; Murray et al., 2015a; Murray et al., 2015b; Murray et al., 2016; Park et al., 2014). Also, this region has been targeted by antibody discovery efforts with some reported success (Lee et al., 2014). We sought to develop a surrogate target strategy (STS) through design of Nav1.7/NavAb chimeric channels incorporating the NaV1.7 ProTx-II binding site into the bacterial channel NavAb as a tool for new molecule discovery and structural studies. Here we report the functional characterization of a chimeric protein, referred to as DII S1-S4, and the development of screening assays using this reagent. The DII S1-S4 chimera will be useful in the search for novel NaV1.7 inhibitors targeting this region.
Materials & Methods

NaVAb chimera construct design. The design focused on replacement of the extracellular loops and significant segment of the transmembrane region of NaVAb with human DII NaV1.7 sequence. To identify regions of interest, sequence alignment (ClustalW with default settings) of NaV1.7 domain II against NaVAb was performed to identify potential splice junctions within the VSD (Figure 1). For the S1-S2 segment NaV1.7 (RefSeq NP_002968) the sequence range selected was ILE743-GLU799 covering the E1 loop region and THR810-ARG830 for the SIII-SIV segment covering E2 loop based on conserved residues at the splice junctions. During the course of this effort, reports based on chimeric strategies to generate crystal structures (Ahuja et al., 2015) and identify toxins (Klint et al., 2015) provided support to the viability of this approach.

Expression of NaVAb chimeras. Recombinant baculovirus (P1) were generated for BirA and all NaVAb variants using the Bac-to-Bac system (Invitrogen). For large scale expressions, high titer baculovirus (P2) was generated by infecting Sf9 insect cells grown in Hyclone serum free media following the Bac-to-Bac protocol. Sf9 insect cells grown in ESF921 media (Expression Systems) using a Wave Bioreactor System 20/50 EHT (GE Healthcare Life Sciences) was used for recombinant protein expression. Sf9 cells cultured to a density of 2.2 x 10^6 cells/ml were co-infected with the P2 recombinant baculovirus (1-1.5 x 10^8 virus particles/ml) of NaVAb chimera and BirA at a ratio of 2:1. The infected cells were maintained at 27°C for 65 hr with bioreactor wave settings suited for 8-10L volumes and the cell pellets were harvested by centrifugation. For expression of non-biotinylated NaVAb, the same protocol was used except that BirA was omitted.

Protein Purification. The Sf9 cell pellet was resuspended and homogenized in a buffer containing 50 mM Tris (pH 8.0), 250 mM NaCl, benzonase (Sigma Aldrich) and protease inhibitor cocktail (Sigma Aldrich). Cells were then lysed by sonication. Total cell lysate was incubated with 1%
n-Dodecyl-β-D-Maltoside (DDM, Anatrace) at 4°C for one h. Cell debris was removed by centrifugation and the supernatant was collected and incubated with anti-Flag M2 affinity resin (Sigma Aldrich) at 4°C for overnight for binding. The protein was eluted with a buffer containing 25 mM Tris (pH 8.0), 250 mM NaCl, 0.1% (w/v) DDM and 100 µg mL⁻¹ 3x Flag peptide (Sigma Aldrich). The eluted protein was concentrated and loaded onto Superdex-200 columns (GE Healthcare) in DPBS buffer containing 0.1% (w/v) DDM. The peak fraction was collected for all the subsequent work. The majority of the chimera constructs behaved similarly during purification. The level of biotinylation was assessed by LCMS and estimated to be at least 50% of the total.

**Cells and reagents.** The HEK cell line stably expressing human Naᵥ1.7 (5N/11S splice form) was purchased from Essen Biosciences (Ann Arbor, MI). ProTx-II and Huwentoxin-IV were purchased from Peptides International (Louisville, KY). [¹²⁵I]ProTx-II was purchased from Perkin Elmer (Waltham, MA). N-terminal 8x His-tagged ProTx-II was a custom synthesis supplied by Smartox Biotechnology (Grenoble, France). Agitoxin-2 was purchased from Alomone Labs (Jerusalem, Israel).

**[¹²⁵I]ProTx-II Binding Assay.** ProTx-II binding to NaᵥAb chimeras or h Naᵥ1.7 channels was determined with a [¹²⁵I]ProTx-II binding assay (Schmalhofer et al., 2008). For NaᵥAb chimeras, Sf9 cells were collected 54 h after transduction with baculovirus by centrifugation for 20 min at 2000 rpm. The cell pellets were washed once with phosphate-buffered saline (PBS) and stored at -70°C. To prepare purified cell membranes, cell pellets were thawed, suspended in homogenization buffer (50 mM HEPES, 0.1% protease inhibitor cocktail (Sigma catalog# P8340), pH=7.4), and homogenized with 25 strokes of a glass Wheaton tissue grinder on ice. This homogenate was centrifuged at 500 x g for 10 min at 4°C on a Sorvall RC6plus centrifuge. The
supernatant was removed and centrifuged at 38500 x g for 60 min at 4°C. This cell pellet was resuspended in assay buffer (50 mM HEPES, 130 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl, pH=7.4), and protein was determined with a Pierce BCA protein assay kit (Thermo Scientific) with BSA used as protein standard. Binding experiments were performed in a 96 deep-well plate with a reaction volume of 100 µl. Non-specific binding was defined by cold ProTx-II (see figure legends for concentrations). All radioligand incubations were performed at room temperature for 6 h. For DII S1-S4 purified protein binding studies, assay buffer contained 0.05% or 0.25% BSA. The binding reaction was terminated by filtration through GF/B filters for membranes and GF/F filters for purified protein, and filters were washed three times with 2 ml of 4°C wash buffer (DPBS with 10% fetal bovine serum for GF/B filters and 50 mM Tris HCl, pH=7.4, for GF/F filters). Filters were presoaked in 0.5% polyethyleneamine. Bound radioactivity on the filters was counted on a Wallac Wizard gamma counter. Specific [125I]ProTx-II binding values were calculated by subtraction of non-specific binding from total binding for [125I]ProTx-II and expressed as mean counts per min (cpm) ± SEM. IC50 values and saturation binding parameters were calculated from fits using GraphPad Prism 7 as described in the legends. Statistical comparisons were performed using unpaired two-tailed t-tests in Prism.

For whole-cell [125I]ProTx-II binding to h NaV1.7, HEK293 cells stably expressing h NaV1.7 were plated in 96 well poly-D-lysine-coated plates at 15,000 cells per well. After 4 days in culture, media was removed and 100 µl of media containing 0.3 nM [125I]ProTx-II was added. Non-specific binding was determined by the inclusion of 600 nM unlabeled ProTx-II. [125I]ProTx-II was incubated with the cells for 6 h at 37°C. At the end of incubation, radioligand was removed and each well was washed twice with 200 µl of wash buffer (Dulbecco’s phosphate-buffer saline, 10% fetal bovine serum). Cells were lysed by addition of 200 µl of 0.2% SDS to each well.
contents of each well was pipetted into a 5 ml plastic tube and counted on a gamma counter. For
$[^{125}\text{I}]$ProTx-II binding to h Na\textsubscript{v}1.7/HEK293 cell membranes, cells stably expressing h Na\textsubscript{v}1.7 channels were grown in T-175 flasks. Cells were detached using detachin, pelleted by centrifugation for 10 min at 1000 rpm, and stored at -80°C. h Na\textsubscript{v}1.7/HEK293 cell membranes were purified as described above for Sf9 membranes.

**Patch clamp electrophysiology.** Membrane currents were recorded using the whole-cell voltage clamp technique. Data were acquired at 50 kHz via an EPC-10 amplifier with Patchmaster software (HEKA Instruments Inc., Bellmore, NY). Glass microelectrodes had resistances of 1-4 MOhms when filled with intracellular solution. Series resistance was kept below 10 MOhms and compensated at least 50%. Leakage subtraction was performed with the P/N method. Methods for recording wild-type Na\textsubscript{v}Ab and chimera channel currents in Sf9 cells were adapted from those described by Gamel El-Din et al. (Gamal El-Din et al., 2013). Briefly, Sf9 cells transduced with baculovirus were cryopreserved 24-31 h post-transduction. On the day of recording, cells were rapidly thawed and plated in Sf-900 II SFM (Thermo Fisher). Recordings were performed between 0.5 h and 5 h after plating. The intracellular solution contained (in mM): 105 CsF, 35 NaCl, 10 EGTA, 10 HEPES, pH 7.4 with CsOH. The external solution was (in mM): 140 NaCl, 2 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, 10 HEPES, pH 7.4 with NaOH. The holding potential was -180 mV. ProTx-II was diluted into external solution supplemented with 0.1% BSA (w:v) and applied to cells by a gravity fed bath perfusion system. For recording Na\textsubscript{v}1.7 currents in HEK293 cells, the intracellular solution contained (in mM): 50 CsCl, 90 CsF, 10 NaF, 2 MgCl\textsubscript{2}, 10 EGTA, 10 HEPES, pH 7.2 with CsOH. The external solution was (in mM): 150 NaCl, 4 KCl, 1 MgCl\textsubscript{2}, 1.8 CaCl\textsubscript{2}, 10 HEPES, 10 glucose, pH 7.4 with NaOH. The holding potential was -110 mV.
Peak membrane currents and tail current amplitudes were measured with Patchmaster software. Conductance was estimated by dividing the peak current at the test potential by the driving force for sodium. For the DII S1-S4 chimera, conductance was estimated by the tail current amplitude upon repolarization from the test potential. The voltage-dependence of activation was determined by fits of the Boltzmann equation of the form: 

$$G(V) = G_{\text{max}} \times \left( \frac{1}{1 + \exp \left( -\frac{(V - V_{1/2})}{k} \right)} \right)$$

where $G_{\text{max}}$ is the maximal conductance, $V_{1/2}$ is the voltage of half-maximal activation and $k$ is the slope. Fitting and plotting of data were performed with IGOR Pro 6 software (WaveMetrics, Inc., Lake Oswego, OR). Data are presented as mean ± SEM.

**Surface Plasmon Resonance (SPR) Assay.** Neutravidin (Pierce/Thermo) was amine-coupled to a Biacore T200 CM5 chip (GE Healthcare) via a standard EDC/NHS kit (GE Healthcare) using these parameters: 40 µg/mL Neutravidin in 10 mM sodium acetate, pH 4.5; 37°C; 15 min contact time at 10 µL/min flow rate. These conditions resulted in the coupling of approximately 22,000 response units (RU) of Neutravidin to the chip surface. Neutravidin coupled surfaces were conditioned with three pulses (30 s) of 1 M NaCl, 40 mM NaOH. Biotinylated NaVAb and chimeras were diluted to 100 nM in PBS with 0.1% w/v n-dodecyl-β-D-maltopyranoside (DDM; Anatrace) and flowed over the Neutravidin surfaces at 25°C on separate flow cells until saturation (typically 1000 to 1200 RU). PBS + 0.1% DDM was used for all subsequent sample dilutions and running buffer at a flow rate of 20 uL/min. Kinetic and steady-state affinity analysis of the resulting binding data was conducted with Biacore T200 Evaluation Software, version 2.0 (GE Healthcare).

**[125I]ProTx-II Scintillation Proximity Assay (SPA).** Scintillation proximity assays were performed in non-treated white 1536-well assay plates (Corning Life Sciences, Corning, NY). Briefly, 1.5 µL of assay buffer (130 mM NaCl, 5.4 mM KCl, 5 mM glucose, 0.8 mM MgCl₂,
0.05% BSA, 0.1% DDM) was dispensed to the assay plate, followed by an equal volume of biotinylated DII S1-S4 protein (3.33 µg/mL). Streptavidin-coated polystyrene imaging beads (1.7 mg/mL; PerkinElmer) were then added, followed by 1.5 µL of radioligand (3.2 nM). Nonspecific binding was determined in the presence of 2 µM unlabeled ProTx-II. The final SPA reaction (6 µL) contained 5 ng DII S1-S4 protein, 2.5 µg/well SPA imaging beads, and 0.8 nM [125I]ProTx-II. DMSO was included in the assay wells at a final concentration of 0.5%. For competition binding experiments, unlabeled ProTx-II was diluted to 4X the final concentration in assay buffer. The sample was diluted 1:3 in assay buffer in a 384-well polypropylene plate (Brooks Automation, Chelmsford, MA) to produce a 16-point dilution series. The final concentration of ProTx-II in the assay ranged from 2500 nM to 0.0017 nM. Unlabeled peptide was preincubated with DII S1-S4 protein for 30 min prior to the addition of SPA beads and radioligand. Following addition of SPA beads and radioligand, the plates were sealed with clear adhesive seals (PerkinElmer) and well luminescence was imaged immediately using a LEADseeker (Amersham, Pittsburgh, PA; instrument settings: 300 s exposure, 2x2 binning). Plates were then incubated at room temperature and imaged at one hour intervals for 12 h. Assay quality and robustness were estimated by calculating the Z’ statistic (Zhang et al., 1999) using the total binding wells (high signal) and nonspecific binding wells (low signal). Raw luminescence data were analyzed by nonlinear regression (4-parameter fit) using GraphPad Prism 7 (La Jolla, CA).
Results

Design, expression and characterization of Nav1.7-NavAb chimeras. We aimed to design a Nav1.7-NavAb chimeric protein that captured key elements of the Nav1.7 DII VSD (Figure 1). Based on sequence alignment we selected a portion of the transmembrane segments including the extracellular loops to represent the target of interest (Nav1.7 DII VSD). The intracellular loops and the remaining segments of the transmembrane region of the template (NavAb) were retained. Chimeric proteins were expressed in Sf9 cells using baculovirus techniques. Two independent constructs were generated (DII S1-S4 and DII S3-S4) for functional characterization and evaluation of suitability as a screening reagent (Supplemental Figure 1).

Electrophysiology of DII chimeras. We explored whether the DII chimeras form functional channels when expressed in Sf9 cells. First, we confirmed that Sf9 cells expressing wild-type NavAb displayed robust inactivating inward currents characteristic of NavAb (Gamal El-Din et al., 2013; Payandeh et al., 2012) while no such currents were detectable in non-transduced Sf9 cells (Supplemental Figure 2). Sf9 cells expressing DII S1-S4 chimera generated currents with distinct characteristics. In response to depolarizations above +50 mV, non-inactivating outward currents were observed and upon repolarization to -180 mV large inward tail currents (Fig. 2A) were seen in 24 out of 39 cells. This current signature is consistent with a voltage-gated channel activating at positive membrane potentials. Tail current analysis estimated a reversal potential close to E_Na (+37 mV, see Methods), consistent with a Na conductance. The voltage-dependence of activation of DII S1-S4 channels estimated from tail current analysis suggested that activation is insignificant below 0 mV with a V_1/2 of +52 mV (Fig. 2B). This activation range is much more positive that wild-type NavAb and wild-type h Nav1.7 (Fig. 2B, Supplemental Table 1). In contrast, DII S3-S4 chimera transduced Sf9 cells generated large, inactivating inward currents.
similar in appearance to Na\textsubscript{V}Ab with a $V_{1/2}$ of activation of -38 mV (Supplemental Figure 3; Supplemental Table 1).

Next, DII S1-S4 chimera currents were tested for their sensitivity to ProTx-II. When measured at the test potential of +60 mV, 10 nM ProTx-II inhibited DII S1-S4 tail current by 94 ± 2\% (n=3). Inhibition was characterized by a positive shift in the voltage-dependence of activation (Fig. 2D). To quantify the effect of ProTx-II on DII S1-S4 activation we chose a concentration of 100 nM, to avoid long incubation times because of the relative instability of these recordings. At 100 nM, ProTx-II shifted activation by +54 mV and decreased maximal conductance by 24\% (Supplemental Figure 4). Hence, ProTx-II is a potent inhibitor of DII S1-S4 channels and acts as a gating modifier, similar to the action of ProTx-II on Na\textsubscript{V}1.7. In contrast, DII S3-S4 channels were not significantly inhibited by 100 nM ProTx-II (5 ± 11\% n=6; data not shown).

**Purification of Na\textsubscript{V}1.7- Na\textsubscript{V}Ab chimeras.** Once expression and function were confirmed, large scale cell cultures (8-10 L) were transduced to yield cell pellets for membrane isolation and protein purification. Wild type Na\textsubscript{V}Ab and chimeras were purified on anti-Flag M2 affinity columns and eluted protein was solubilized in detergent (0.1\% (w/v) DDM). SDS-PAGE analysis demonstrated proteins with the predicted molecular mass (22 kDa, Supplemental Figure 5). Protein purity was estimated to be greater than 90%.

**[125I]ProTx-II binding.** Radiolabeled ProTx-II binding was also used to test for ProTx-II sensitivity of DII chimeras. A baseline was established with ProTx-II binding to human Na\textsubscript{V}1.7. As expected, HEK293 cells stably expressing wild-type human Na\textsubscript{V}1.7 channels displayed robust specific binding of [125I]ProTx-II (Fig. 3A). In membrane preparations from these same cells, no specific binding of [125I]ProTx-II was detectable. The average specific binding window in cells was 5.6-fold compared to 1.1-fold in membranes (p<0.05). In membrane preparations from Sf9
cells expressing the DII S1-S4 chimera, significant specific binding of $[^{125}\text{I}]$ProTx-II was apparent (Fig. 3B). Interestingly, non-transduced Sf9 cells displayed a lower but measurable total $[^{125}\text{I}]$ProTx-II binding which was similar to that observed in cells expressing either wild-type NavAb or the DII S3-S4 chimera (Fig. 3B). In all cases this binding was largely displaced by cold ProTx-II, suggesting potential alternate site(s) of specific interaction on the Sf9 membrane. Nevertheless, the highest specific $[^{125}\text{I}]$ProTx-II binding was observed in Sf9 membranes expressing the DII S1-S4 chimera (7.2-fold compared to about 4-fold for Sf9, NavAb, or DII S3-S4; $p<0.05$), suggesting direct binding to the DII S1-S4 chimeric protein. Given this observation, we purified DII S1-S4 protein and tested for $[^{125}\text{I}]$ProTx-II binding. Specific $[^{125}\text{I}]$ProTx-II binding was observed with purified DII S1-S4 protein in detergent with a clear dependence on the amount of chimeric protein (Fig. 3C). The highest specific binding was observed at 0.3 µg protein (8.8-fold, $p<0.05$ compared to the other protein concentrations). No specific $[^{125}\text{I}]$ProTx-II binding was detectable with purified DII S3-S4 protein (Supplemental Figure 6).

The $[^{125}\text{I}]$ProTx-II binding data with Sf9 membranes and purified proteins suggested that the DII S1-S4 chimera but not the DII S3-S4 chimera reconstituted the high affinity ProTx-II binding site. To characterize this binding further, we performed competition binding experiments on purified DII S1-S4 protein with cold ProTx-II and a related peptide, Huwentoxin-IV (Fig. 3D). Cold ProTx-II displaced specific $[^{125}\text{I}]$ProTx-II binding with an $IC_{50}$ of 36 nM whereas Huwentoxin-IV was weaker ($IC_{50}$ of 662 nM) consistent with literature (Xiao et al., 2010). Saturation binding experiments of $[^{125}\text{I}]$ProTx-II binding to the DII S1-S4 chimera estimated a $K_d$ of 9.4 nM (Supplemental Figure 7).

**SPR Characterization.** In order to explore the kinetics of binding interactions between the toxins and DII chimeras, biotinylated versions of the purified NavAb, DII S3-S4, and DII S1-S4 proteins
were captured onto a Biacore CM5 chip derivatized with Neutravidin. His-tagged ProTx-II, Huwentoxin-IV, and Agitoxin-2 were then flowed over these surfaces in order to detect binding interactions. As shown in Figure 4, ProTx-II and Huwentoxin-IV bound specifically to the DII S1-S4 chimera, but not to NavAb or DII S3-S4 chimera. Agitoxin-2 did not bind to any of these surfaces, as expected (data not shown). In order to test the reproducibility of this system, concentration series of the ProTx-II and Huwentoxin-IV were flowed over these surfaces in duplicate. As shown in Figure 4C and Figure 4D, concentration-dependent binding was detected for both toxins, and sample duplicates demonstrated good reproducibility of the binding signal. These binding data did not fit well to a 1:1 binding model, so on- and off-rates could not be accurately determined. Fitting with a steady-state binding model yielded apparent affinities of 15 nM for ProTx-II and 125 nM for Huwentoxin-IV consistent with the rank order reported in literature for WT human Nav1.7 (Xiao et al., 2010).

**SPA Assay.** Given the robust binding of ProTx-II to purified DII S1-S4 protein detected by filter binding and SPR, we next sought to develop a Scintillation Proximity Assay (SPA) for screening. Biotinylated DII S1-S4 protein was immobilized on streptavidin-coated SPA beads and incubated with $^{[125I]}$ProTx-II. Initial studies were performed in 384-well format and subsequently the assay was optimized for 1536-well plates. Optimization for protein and $^{[125I]}$ProTx-II usage yielded an assay with suitable sensitivity to competition by cold ProTx-II ($IC_{50} = 107$ nM; Fig. 5A). The $IC_{50}$ of competition by cold ProTx-II peptide was stable for at least 8 h (average $IC_{50}$: 118 ± 12 nM; range: 99 nM to 135 nM) (Fig. 5B). The reduced potency of ProTx-II in this assay compared to filter binding was likely due to ligand depletion in the low volume format (6 µL). The signal-to-background ratio increased slightly over the first hour of incubation, was then stable for 10 h at room temperature (average: 7.0 ± 0.43) and decreased to the approximate $t = 0$ level by 12 h (Fig.
Assay robustness, as indicated by the Z’ statistic, was stable over the course of the experiment (average Z’: 0.62 ± 0.03). As expected, the assay signal was also sensitive to Huwentoxin IV with reduced potency (IC$_{50}$ = 1.3 µM) compared to ProTx-II (data not shown).

**Discussion**

Nav1.7 is a target of high importance for the development of novel analgesics based on human genetics linking this channel to pain perception. Two sites on Nav1.7 are known to yield selective inhibitors of channel function. Small molecule sulfonamides bind to VSD4, and lock the VSD in the activated conformation, leading to channel inactivation (Ahuja et al., 2015; McCormack et al., 2013). The other site is on VSD2, where gating modifier peptides such as ProTx-II bind (Schmalhofer et al., 2008). ProTx-II inhibits Nav channels by a distinctly different mechanism - it traps the voltage sensor in the resting state, making it more difficult for voltage to open the channel (Sokolov et al., 2008). While both sites are of significant interest, it is not yet known if these mechanistically distinct approaches to channel inhibition provide any advantage from a therapeutic standpoint. Nevertheless, efforts to thoroughly explore each of these pharmacophores have been rigorous. Here we describe the characterization of a novel chimeric protein comprising the human Nav1.7 DII S1-S4 region with the bacterial Nav channel, NavAb, which is suitable for high throughput screening for novel agents targeting the VSD2 site.

We find the DII S3-S4 and DII S1-S4 chimeras’s form functional channels with unique properties. Most notably, in comparison to NavAb a positive shift in activation of 55 mV for DII S3-S4 and 145 mV for DII S1-S4 respectively. While the DII S3-S4 represents an activation profile closer to that observed for WT-NavAb the DII S1-S4 profile renders it such that channel opening is negligible at 0 mV. This activation profile uniquely qualifies the DII S1-S4 for use in identification of modulators that target primarily target the resting state of the channel. We
speculate the large shift in activation profiles originate from changes in the pairing environment of the gating charges spanning the S4 segment of the DII VSD’s and can vary dependent on the template used. A right shift in the activation potential for the DII Kv2.1-rat Na\textsubscript{v}1.2 chimera compared to the template reference (Kv2.1) was also observed in previously reported studies (Bosmans et al., 2008).

Previous studies have found that transferring a section of the helix-turn-helix motif covering E3 loop of Na\textsubscript{v}1.7 DII S3-S4 to Kv2.1 conferred ProTx-II sensitivity to the channel chimera (Bosmans et al., 2008). Additionally, mutation of individual residues in the DII S3-S4 region (e.g. F813 and E818) reduce ProTx-II sensitivity (Schmalhofer et al., 2008; Xiao et al., 2010). Likewise, a residue (L833) in DII S3 of Na\textsubscript{v}1.2 is important for ProTx-II inhibition (Sokolov et al., 2008). In contrast, mutation of residues in DII S1-S2 of Na\textsubscript{v}1.5 did not significantly affect ProTx-II inhibition (Smith et al., 2007). We find that transfer of Na\textsubscript{v}1.7 DII S3-S4 helix-turn-helix motif to Na\textsubscript{v}Ab did not yield channels sensitive to ProTx-II. One possible reason for the difference in observation could have been a result of the variation in the inserted sequence. To reconstitute high affinity ProTx-II binding, the helix-turn-helix motif of Na\textsubscript{v}1.7 DII S1-S2 was also required. These results suggest that in the context of Na\textsubscript{v}Ab template additional residues of Na\textsubscript{v}1.7 DII S1-S2 are likely important for ProTx-II binding.

Gating modifier peptides stabilize the resting conformation and affinity is reduced when the voltage sensor is in the activated conformation (Tilley et al., 2014). Hence, the positive activation profile may have allowed for detection of high affinity ProTx-II binding in membranes and with purified protein. Despite the high affinity binding observed with the purified DII S1-S4 chimera (K\textsubscript{d} = 30 nM), the binding is weaker than that observed with wild-type human Na\textsubscript{v}1.7 expressed in HEK293 cells (K\textsubscript{d} = 0.3 nM) (Schmalhofer et al., 2008). The lower binding affinity
observed with DII S1-S4 may result from a combination of alternate VSD conformations and/or the influence of the detergent resulting in significantly different environment compared to live HEK293 cells expressing wild-type Na\textsubscript{v}1.7 (Marheineke et al., 1998). Gating modifier peptides are known to partially partition into membranes (Lee and MacKinnon, 2004; Mihailescu et al., 2014; Milescu et al., 2007) and ProTx-II clearly interacts with phospholipids (Henriques et al., 2016; Smith et al., 2005). Conversely, VSD interactions with lipid can influence toxin binding (Milescu et al., 2009). Auxiliary subunits are also known to influence gating modifier action on Na\textsubscript{v} channels (Wilson et al., 2011). Thus, the affinity of DII S1-S4 for ProTx-II is likely impacted by the expression/reconstitution system employed here. Alternatively, other residues present in Na\textsubscript{v}1.7 VSD2 but not included in the DII S1-S4 chimera may be important for ProTx-II binding either through direct interaction or providing key structural features of the VSD. If so, further refinements of the DII S1-S4 chimera may improve ProTx-II binding affinity. Also, it is important to note that the other VSDs of Na\textsubscript{v}1.7 appear to interact with ProTx-II (Bosmans et al., 2008; Xiao et al., 2014; Xiao et al., 2010) which would not be captured in the chimera reported here.

Purified DII S1-S4 protein in detergent capable of high affinity ProTx-II binding opens several possibilities for use in discovery efforts. For example, the protein may be useful as an antigen in monoclonal antibody discovery, screening reagent to identify molecules such as toxins, adnectins, synthetic peptides, milla molecules and small molecules. Screening approaches based on detection of binding to the chimera (and not wild-type Na\textsubscript{v}Ab) may yield molecules that interact specifically with the transplanted residues of DII S1-S4. Furthermore, traditional screening approaches based on competition of \textsuperscript{[125I]}ProTx-II binding are feasible. The ability of purified DII S1-S4 to bind ProTx-II affords several opportunities for assay development not possible with crude membrane preparations containing channels at relatively low density. For example, we were able
convert and miniaturize the $[^{125}\text{I}]$ProTx-II binding assay to a SPA format in 1536 wells. The homogenous assay is sufficiently robust to support high throughput screening, with a large window that is stable over several hours. We note that the observed IC$_{50}$ of cold ProTx-II peptide in the SPA assay is approximately 5-fold greater than that observed in filter binding experiments. The final SPA conditions required 5 ng of chimeric channel to yield an acceptable assay which corresponds to a 27-fold molar excess of chimeric protein over the radioligand. The lower potency of cold ProTx-II in the SPA format may be the result of ligand depletion. The effect of ligand depletion on measured IC$_{50}$s ligand depletion as has been documented for other SPA assays (Carter et al., 2007). Scintillation proximity assays for other ion channels have been based on membrane preparations rather than purified protein (Hui et al., 2005; Zhang et al., 2006). One caution common to any chimera approach is the need to test any hits identified from a screen for activity against the wild-type Na$_{V}$1.7 in a functional assay.

Surface plasmon resonance (SPR) is a powerful method to study protein-peptide interactions in detail. Alpha-scorpion toxin binding to isolated Na$_{V}$1.2 VSD4 can be detected by SPR (Martin-Eauclaire et al., 2015). Here we show that an SPR assay based immobilization of an entire chimeric channel (DII S1-S4) is sensitive to ProTx-II binding. No binding was detectable with immobilized wild-type Na$_{V}$Ab or the DII S3-S4 chimera, confirming that the SPR signal reflects a specific interaction with the Na$_{V}$1.7 DII chimera. Furthermore, the estimated K$_{d}$ based on SPR aligns well with $[^{125}\text{I}]$ProTx-II binding competition and electrophysiology studies. A robust SPR assay for the DII S1-S4 chimera has several potential uses. For example, such an assay is ideal for confirming direct, specific interaction of novel molecules identified by screening with the chimera. Also, monoclonal antibodies identified from either traditional hybridoma approaches or phage panning can be tested for binding. Another obvious use of such an assay is the
biophysical characterization of the interaction of molecules with the chimeric channel, including kinetic and affinity estimates.

In summary, here we report a functional NaV1.7 DII chimera with the bacterial channel NaVAb. The chimera forms functional channels and is sensitive to the NaV1.7 gating modifier peptide, ProTx-II. We demonstrate the utility of this chimera by engineering both SPA and SPR assays with purified protein. The DII S1-S4 chimera will prove useful for the discovery and characterization of novel molecules targeting this important functional domain of NaV1.7. Furthermore, this chimera approach may have broad applicability for enabling screening at other membrane protein targets of pharmaceutical interest.
Authorship Contributions

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References


Figure Legends

**Figure 1. Design of Nav1.7-NavAb chimeras.** Sequence alignment of the S1-S2 (top) and S3-S4 (bottom) regions of human Nav1.7 domains DII with NavAb. The splice junctions chosen for creation of chimeras are designated by the anchor symbols.

**Figure 2. [125I]ProTx-II binding of Nav1.7-NavAb chimeras.** A. [125I]ProTx-II binding to hNav1.7 channels expressed in HEK293 cells. For whole-cell binding (left), 15,000 cells per well in a 96-well plate were incubated with 0.36 nM [125I]-ProTx-II. Non-specific (NS) binding was defined by addition of 0.6 µM cold ProTx-II. Bars represent the mean ± SEM cpm s, n=6. For membranes (right), 50 µg h Nav1.7 HEK membranes were incubated with 0.15 nM [125I]-ProTx-II. Non-specific binding was defined by addition of 1 µM cold ProTx-II. Bars represent the mean ± SEM cpm s, n=3. B. 1 µg Sf9 membranes from non-transduced cells (Sf9) or cells transduced with either wt NavAb, DII S3-S4 chimera or DII S1-4 chimera were incubated with 1.2 nM [125I]-ProTx-II. Non-specific binding was defined by addition of 20 µM cold ProTx-II. Bars represent the mean ± SEM cpm s, n=3. C. Purified DII S1-S4 protein (0.003-3.0 µg) was incubated with 1.9 nM [125I]-ProTx-II. Non-specific binding was defined by addition of 0.6 µM cold ProTx-II. Bars represent the mean ± SEM cpm s, n=3. D. Percent inhibition of specific [125I]-ProTx-II (1.1 nM) binding to purified DII S1-S4 protein (0.3 µg) by cold ProTx-II (circles) and Huwentoxin-IV (squares). Non-specific binding was defined by addition of 2 µM cold ProTx-II. Symbols represent the mean ± SEM inhibition of three independent experiments measured in triplicate. Estimated IC50s from the fits are 36 nM (ProTx-II) and 662 nM (Huwentoxin-IV).
Figure 3. DII S1-S4 chimera forms functional, ProTx-II sensitive channels. A. Representative recording from a Sf9 cell transduced with BacMam encoding DII S1-S4 chimera. Currents were evoked by 20 msec step depolarizations between -140 mV and +120 mV. For clarity, only step depolarizations between -20 mV and +100 mV in 20 mV increments are shown. B. Plot of the mean ± SEM relative conductance of NaVAb (circles, n=9), h NaV1.7 (diamonds, n=12) and DII S1-S4 chimera (squares, n=9) versus membrane potential. The solid lines are fits of the Boltzmann equation to the data. Parameters of the fits are given in Table 1. C. Representative recording from a Sf9 cell transduced with BacMam encoding DII S1-S4 chimera. Currents were evoked by a 20 msec step depolarization to +60 mV in control and in the presence of 10 nM ProTx-II. D. Plot of the relative conductance versus membrane potential for the recording in panel C in control (circles) and 100 nM ProTx-II (triangles). Conductance was estimated from the tail current amplitude and normalized to the control amplitude at the +120 mV test potential.

Figure 4. Surface plasmon resonance (SPR) binding assay for DII S1-S4 chimera. A and B. Biacore T200 SPR sensorgrams showing binding profiles of his-tagged ProTx-II (A; 100 nM) or Huwentoxin-IV (B; 1000 nM) to biotinylated NaVAb (black), DII S3-S4 (blue) or DII S1-S4 (red). C. Binding of a duplicate 2-fold concentration series (7.8 nM to 1000 nM) of ProTx-II to DII S1-S4. Each color indicates a separate concentration in the series. D. Binding of a duplicate 2-fold concentration series (7.8 nM to 1000 nM) of Huwentoxin-IV (62.5 nM to 1000 nM) to DII S1-S4. Each color indicates a separate concentration in the series. Blank buffer injections as well as background binding to a Neutravidin-only flow cell have been subtracted from all displayed sensorgrams.
Figure 5. Scintillation proximity assay (SPA) with DII S1-S4 chimera in 1536-well format.

A. Competition of the SPA luminescence signal (relative light units, RLU) by cold ProTx-II after a 4 h incubation. Symbols are the mean ± SD of four replicate wells. The solid line is a 4-parameter fit with an IC₅₀ of 107 nM and Hill slope of 0.95. B. Plot of the pIC₅₀ of competition by cold ProTx-II versus time. C. Plot of the signal-to-background ratio versus time. D. Plot of the assay Z' statistic versus time. The signal-to-background ratio and Z' statistic were calculated using 2 columns each (32 wells) for the assay high and low signals.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5