Correction

NEUROSCIENCE, CHEMISTRY

The authors note that the title appeared incorrectly. The title should instead appear as “Marked difference in saxitoxin and tetrodotoxin affinity for the human nociceptive voltage-gated sodium channel (Na1.7).” The online version has been corrected.

www.pnas.org/cgi/doi/10.1073/pnas.1220041110
Marked difference in saxitoxin and tetrodotoxin affinity for the human nociceptive voltage-gated sodium channel (Na\textsubscript{v}1.7)

James R. Walker\textsuperscript{a}, Paul A. Novick\textsuperscript{a}, William H. Parsons\textsuperscript{a}, Malcolm McGregor\textsuperscript{b}, Jeff Zablocki\textsuperscript{c}, Vijay S. Pande\textsuperscript{d,e}, and J. Du Bois\textsuperscript{a,1}

Departments of \textsuperscript{a}Chemistry, \textsuperscript{b}Structural Biology, and \textsuperscript{c}Computer Science, Stanford University, Stanford, CA 94305-5080; \textsuperscript{d}Accelrys, Inc., San Diego, CA 92121; and \textsuperscript{e}Department of Medicinal Chemistry, Gilead Sciences Inc., Foster City, CA 94404

Edited by Michael D. Cahalan, University of California, Irvine, CA, and approved September 14, 2012 (received for review May 9, 2012)

Human nociceptive voltage-gated sodium channel (Na\textsubscript{v}1.7), a target of significant interest for the development of antinociceptive agents, is blocked by low nanomolar concentrations of (−)-tetrodotoxin (TTX) but not (+)-saxitoxin (STX) and (+)-gonyautoxin-II (GTX-III). These findings question the long-accepted view that the 1.7 isoform is both tetrodotoxin– and saxitoxin-sensitive and identify the outer pore region of the channel as a possible target for the design of Na\textsubscript{v}1.7-selective inhibitors. Single- and double-point amino acid mutagenesis studies along with whole-cell electrophysiology recordings establish two domain III residues (T1398 and I1399), which occur as methionine and aspartate in other Na\textsubscript{v} isoforms, as critical determinants of STX and gonyautoxin-II binding affinity. An advanced homology model of the Na\textsubscript{v} pore region is used to provide a structural rationalization for these surprising results.

SCN9A | guanidinium toxin

The propagation of electrical signals in nerve cells is mediated by an ensemble of proteins, among which voltage-gated sodium ion channels (Na\textsubscript{s}) play a leading role. In mammalian cells, functional Na\textsubscript{s} are expressed as a pore-forming \(\alpha\)-subunit with four nonidentical repeats within a single polypeptide and one or two auxiliary \(\beta\)-proteins (1); 10 gene loci have been identified that encode the 10 unique isoforms of the \(\alpha\)-subunit (Na\textsubscript{v}1.1–1.9 and Na\textsubscript{v}5). Mutations of the Na\textsubscript{v}1.7 isoform are known to result in disease states, with hyperfunctioning mutants conferring increased pain sensitivity to patients and hypofunctioning mutations resulting in complete insensitivity to pain (2, 3). These findings have generated a tremendous interest in Na\textsubscript{v}1.7 as a clinical target for the treatment of pain (4–7). Designing small-molecule modulators of specific Na\textsubscript{v} subtypes, however, is challenged by the high sequence homology between the different \(\alpha\)-subunits and the lack of detailed structural information for any mammalian Na\textsubscript{v} channel (8, 9).

Nature has provided a collection of topologically unique small molecules that bind with high affinity and varying degrees of isoform selectivity to the Na\textsubscript{v} family of proteins. The guanidinium toxins, exemplified by the fugu poison (−)-tetrodotoxin (TTX), are one such class of agents that blocks Na\textsubscript{v} influx by lodging in the outer mouth of the \(\alpha\)-subunit defined by the pore loops (site 1) (10). Other guanidine-derived natural products, namely (+)-saxitoxin (STX) (11) and the gonyautoxins (known collectively as paralytic shellfish poisons), are proposed to act analogously (12, 13). Early work to characterize Na\textsubscript{v}1.7 (12) demonstrated that in CHO cells the activity of TTX for Na\textsubscript{v}1.7 is 702 ± 53 nM, a 250-fold difference in potency. Structurally related GTX-III also displays a significant reduction in potency against hNa\textsubscript{v}1.7 compared with TTX, STX, and gonyautoxin-III against recombinant hNa\textsubscript{v}1.7 α-subunit expressed in CHO cells. For comparative purposes, an identical series of measurements was made using rat Na\textsubscript{v}1.4 (CHO). These data reveal striking and unexpected differences in affinity for TTX vs. STX and GTX-III against the two isoforms. As shown in Fig. 1 and Table 1, TTX blocks rNa\textsubscript{v}1.4 with an IC\textsubscript{50} value of 2.8 ± 0.1 nM consistent with reported literature values (12). By contrast, the IC\textsubscript{50} value of STX for hNa\textsubscript{v}1.7 is 702 ± 53 nM, a 250-fold difference potency. Analogue experiments performed with TTX confirm that the affinity of this toxin for both channel isoforms is comparable (hNa\textsubscript{v}1.7: 18.6 ± 1.0 nM; rNa\textsubscript{v}1.4: 17.1 ± 1.2 nM).

Comparative analyses of the primary sequences thought to comprise the pore loop regions of rNa\textsubscript{v}1.4 and hNa\textsubscript{v}1.7 have led to the identification of two amino acid variations found in repeat III of the \(\alpha\)-subunit, M1240T and D1241I (rNa\textsubscript{v}1.4 numbering). A BLAST search of the National Center for Biotechnology Information database reveals this double variation to be unique to human and other primate Na\textsubscript{v}1.7 (Table 2 and Table S1); no other mammalian Na\textsubscript{v} isoforms have both threonine and isoleucine at positions 1240 and 1241.

Results

Given the intense interest in human Na\textsubscript{v}1.7 as a target for pain (4–7, 15) and growing interest in clinical applications of guanidinium toxins as antinociceptive agents (16–23), we began a series of electrophysiology recordings with TTX, STX, and gonyautoxin-III (GTX-III) against recombinant hNa\textsubscript{v}1.7 α-subunit expressed in CHO cells. For comparative purposes, an identical series of measurements was made using rat Na\textsubscript{v}1.4 (CHO). These data reveal striking and unexpected differences in affinity for TTX vs. STX and GTX-III against the two isoforms. As shown in Fig. 1 and Table 1, TTX blocks rNa\textsubscript{v}1.4 with an IC\textsubscript{50} value of 2.8 ± 0.1 nM consistent with reported literature values (12). By contrast, the IC\textsubscript{50} value of STX for hNa\textsubscript{v}1.7 is 702 ± 53 nM, a 250-fold difference potency. Structurally related GTX-III also displays a significant reduction in potency against hNa\textsubscript{v}1.7 compared with rNa\textsubscript{v}1.4 (1.513 ± 55 vs. 14.9 ± 2.1 nM, respectively). Analogous experiments performed with TTX confirm that the affinity of this toxin for both channel isoforms is comparable (hNa\textsubscript{v}1.7: 18.6 ± 1.0 nM; rNa\textsubscript{v}1.4: 17.1 ± 1.2 nM).

Comparative analyses of the primary sequences thought to comprise the pore loop regions of rNa\textsubscript{v}1.4 and hNa\textsubscript{v}1.7 have led to the identification of two amino acid variations found in repeat III of the \(\alpha\)-subunit, M1240T and D1241I (rNa\textsubscript{v}1.4 numbering). A BLAST search of the National Center for Biotechnology Information database reveals this double variation to be unique to human and other primate Na\textsubscript{v}1.7 (Table 2 and Table S1); no other mammalian Na\textsubscript{v} isoforms have both threonine and isoleucine at positions 1240 and 1241.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

1To whom correspondence should be addressed. E-mail: jdubois@stanford.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1206952109/-/DCSupplemental.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

1To whom correspondence should be addressed. E-mail: jdubois@stanford.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1206952109/-/DCSupplemental.
structurally homologous sites in the polypeptide. Accordingly, a series of reciprocal single- and double-point mutants of rNa\textsubscript{V\textsubscript{1.4}} and hNa\textsubscript{V\textsubscript{1.7}} was generated to test whether one or both of these amino acids were responsible for altering the binding affinity of STX and GTX-III.

Three mutant forms of rNa\textsubscript{V\textsubscript{1.4}} and one mutant of hNa\textsubscript{V\textsubscript{1.7}} have been prepared and were successfully expressed in CHO cells and showed current densities similar to WT. Electrophysiology recordings give compelling evidence that M\textsubscript{\rightarrow}T and D\textsubscript{\rightarrow}I substitutions are important modulators of STX/GTX-III affinity to Na\textsubscript{\textsc{v}}. As noted in Table 1, 1-aa mutations in rNa\textsubscript{V\textsubscript{1.4}} result in only moderate increases in measured IC\textsubscript{50} values for both STX and GTX-III. The potency of STX and GTX-III to the double mutant, rNa\textsubscript{V\textsubscript{1.4}} M1240T-D1241I, however, is greatly reduced (1,153 ± 60 nM and 1,084 ± 46 nM, respectively). To further examine the combined effect of M1240 and D1241 on STX/GTX-III binding, commensurate mutations were made to T1398 and I1399 in hNa\textsubscript{V\textsubscript{1.7}}. Against hNa\textsubscript{V\textsubscript{1.7}} T1398M-I1399D, the IC\textsubscript{50} values for STX and GTX-III were determined to be 2.3 ± 0.2 and 22 ± 2.9 nM, respectively. The measured affinities for

<table>
<thead>
<tr>
<th>Toxin</th>
<th>rNa\textsubscript{V\textsubscript{1.4}}</th>
<th>hNa\textsubscript{V\textsubscript{1.7}} T1398M-I1399D*</th>
<th>rNa\textsubscript{V\textsubscript{1.4}} M1240T</th>
<th>rNa\textsubscript{V\textsubscript{1.4}} D1241I</th>
<th>rNa\textsubscript{V\textsubscript{1.4}} M1240T-D1244I</th>
</tr>
</thead>
<tbody>
<tr>
<td>STX</td>
<td>702 ± 53</td>
<td>2.3 ± 0.2</td>
<td>2.8 ± 0.1</td>
<td>73 ± 2.6</td>
<td>53 ± 4.6</td>
</tr>
<tr>
<td>GTX-III</td>
<td>1,513 ± 55</td>
<td>22 ± 2.9</td>
<td>14.9 ± 2.1</td>
<td>228 ± 4.3</td>
<td>38 ± 1.8</td>
</tr>
<tr>
<td>TTX</td>
<td>18.6 ± 1.0</td>
<td>5.0 ± 0.9</td>
<td>17.1 ± 1.2</td>
<td>466 ± 42</td>
<td>8.7 ± 0.8</td>
</tr>
</tbody>
</table>

IC\textsubscript{50} values were determined by fitting /\textsubscript{Io} vs. log[toxin], where /\textsubscript{Io} = normalized current, to a Langmuir isotherm (toxin concentrations ≥ 4). Currents were elicited by 10-ms step depolarizations from a holding potential of −100 to 0 mV.

*T1398 and I1399 in hNa\textsubscript{V\textsubscript{1.7}} are structurally homologous with M1240 and D1241 in rNa\textsubscript{V\textsubscript{1.4}}.

---

Table 1. Measured IC\textsubscript{50} values ± SD (nanomolar; n ≥ 3 cells) for STX, GTX-III, and TTX against WT and mutant Na\textsubscript{\textsc{v}}s.
TTX against all three rNa,1.4 mutants differs considerably from the other two toxins. A marked increase in TTX IC$_{50}$ is observed only for the single-point mutant M1240T, which was previously noted in the work by Jost et al. (24). Compared with TTX binding against WT rNa,1.4, the measured IC$_{50}$ value for toxin block of rNa,1.4 D1241I is slightly decreased (8.7 ± 0.8 nM), whereas the IC$_{50}$ value for rNa,1.4 M1240T D1241I is increased by fivefold (90 ± 4.7 nM). As indicated by these data, changes to these repeat III residues in the channel pore do not alter tightly TTX binding. This conclusion is affirmed by experiments that measured TTX block of hNa,1.7 T1398M-I1399D, for which the TTX IC$_{50}$ value is 5.0 ± 0.9 nM.

In an attempt to rationalize the observed differences in potency between STX, GTX-III, and TTX against both WT and mutant Na$_s$, we performed a series of docking studies with homology models of the pore structure. For the purpose of this analysis, two reported Na$_s$, homology models and one that we developed were evaluated (25, 26). All three models use a prokaryotic K$^+$ channel (KcsA, MthK, or K$_s$AP) as a starting template. Our model is comprised of the pore helix, the P-loop, and the S6 helix from each domain (Fig. 2 A and B). The putative toxin binding site is formed by residues 400–404, 755–758, 1,239–1,242, and 1,530–1,533. Docking models of STX and TTX were created against available amino acid mutagenesis data (27), which show E403, E758, M1240, and D1532 to be critical for toxin block (Table S2). The model developed by Zhorov and our model are both capable of recapitulating the strong binding interactions between the toxins and these select residues (Fig. S2). The two models have similar positioning of backbone atoms (rmsd = 1.5 Å for backbone atoms only); however, significant conformational differences are apparent in amino acid side chain atoms (rmsd = 3.2 Å for nonbackbone atoms). An additional constraint is present in the Zhorov structure that fixes an interaction between the 7,8,9 guanidinium of STX and D400. Mutagenesis data (D400A) show that this aspartate residue does not contribute appreciably to STX binding (Table S2). For the purpose of introducing a minimum number of constraints to our model, specific contact between the guanidinium toxin and domain I Y401 was not included during model construction. Docking poses for TTX, STX, and GTX-III, however, show the aromatic ring of the tyrosine residue positioned within 3.5 Å of the respective guanidinium moiety. Such findings, particularly with TTX, are consistent with experimental data that post a guanidinium cation–π interaction with Y401 (28, 29).

Given recent reports of X-ray crystal structures for bacterial voltage-gated sodium channels Na$_s$ (8) and Na$_r$Rh (9), we have examined one of these channels (Na$_s$Ab) as a template for the eukaryotic sodium channel. Alignment of the primary sequences of Na$_s$Ab and Na,1.4 was performed to mark the indices on Na$_s$Ab corresponding to the P-loop region and toxin binding site (Table S2). Analysis of the Na$_s$Ab X-ray structure reveals that outer vestibule loop amino acids E403, E758, M1240, and D1532 (Na,1.4 numbering) do not face the pore lining and thus, would be precluded from interacting with bound toxins. A more thorough evaluation of Na$_s$Ab as a template for eukaryotic Na,1.7 has been recently reported (30). Sequence homology between the prokaryotic and eukaryotic channels in the pore loop region is low, and the former is not inhibited by TTX (31). Adjustment of the sequence alignment by inserting an additional amino acid in the P-loop of Na,1.4 is needed to produce a model that faithfully recapitulates TTX binding data. From this analysis, the conclusions of which are reinforced by our computational experiments, we conclude that the structure of Na$_s$Ab is no more accurate a starting template for construction of a Na,1.4 homology model than KcsA or K$_s$AP.

Comparing structures of homology models of hNa,1.7, rNa,1.4, and the four mutant proteins reveals the influence of mutations on the binding pocket size, shape, and electrostatic surface at site 1. The site 1 binding pocket in the outer vestibule of hNa,1.7 is larger than the binding pocket of rNa,1.4, owing primarily to the replacement of the methionine residue with the sterically smaller threonine (Fig. 2 C and D). In Fig. 2 E–H, the electrostatic surface potentials predicted for rNa,1.4, hNa,1.7 T1398M-I1399D, rNa,1.4 M1240T-D1241I, and hNa,1.7, respectively, are shown. In the region of residues 1,240 and 1,241, the surface electrostatic potential of both rNa,1.4 and hNa,1.7 T1398M-I1399D varies in the region between −11 and −16 kT, whereas the surface potential of hNa,1.7 and rNa,1.4 M1240T-D1241I is a more positive −3 to −8 kT. Analysis of the single-point mutants rNa,1.4 M1240T and D1241I reveals that the effect of these amino acid substitutions is cumulative, with both threonine and isoleucine contributing to a larger, less negative toxin binding site (compared with WT rNa,1.4) (Fig. S3).

Analysis of the steric volume and electrostatic surface potential for each toxin highlight marked differences between the three natural products (Fig. 3). The molecular volumes for both STX and TTX are similar (251 and 252 Å$^3$, respectively), despite their disparate molecular shapes; GTX-III is 54 Å$^3$ larger as a result of the C11 sulfate moiety. At physiological pH, TTX and GTX-III are both monocations, whereas STX exists entirely in its diprotonated form. Accordingly, large differences between the three toxins are noted in the calculated electrostatic surface potentials. Although the guanidinium group of TTX is positive at the molecular surface, the rest of the molecule is largely neutral with small regions of charge density. This finding is in contrast to STX, where the electrostatic potential is above 4 kT across the entire surface of the molecule, except for the carbamate side chain. The addition of the sulfate on GTX-III partially neutralizes the surface potential of the guanidinium group and creates two negative regions surrounding the C11 sulfate and C13 carbamate.

### Table 2. Pore-forming sequence alignment of Na,1.7 from select primate and nonprimate species

<table>
<thead>
<tr>
<th>Animal</th>
<th>Domain I</th>
<th>Domain II</th>
<th>Domain III</th>
<th>Domain IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>RLMTQDYWEN</td>
<td>RVLCGEWIET</td>
<td>VATFKGWTHI</td>
<td>ITTSGWGVDGL</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>RLMTQDYWEN</td>
<td>RVLCGEWIET</td>
<td>VATFKGWTHI</td>
<td>ITTSGWGVDGL</td>
</tr>
<tr>
<td>Rhinoceros monkey</td>
<td>RLMTQDYWEN</td>
<td>RVLCGEWIET</td>
<td>VATFKGWTHI</td>
<td>ITTSGWGVDGL</td>
</tr>
<tr>
<td>Nonprimate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>RLMTQDYWEN</td>
<td>RVLCGEWIET</td>
<td>VATFKGWMDI</td>
<td>ITTSGWGVDGL</td>
</tr>
<tr>
<td>Rat</td>
<td>RLMTQDYWEN</td>
<td>RVLCGEWIET</td>
<td>VATFKGWMDI</td>
<td>ITTSGWGVDGL</td>
</tr>
<tr>
<td>Cow</td>
<td>RLMTQDYWEN</td>
<td>RVLCGEWIET</td>
<td>VATFKGWMDI</td>
<td>ITTSGWGVDGL</td>
</tr>
</tbody>
</table>

The domain III M→T, D→I variation is underlined and observed only in primate Na,1.7. Selectivity filter amino acids, DEKA, are bold. A complete list of Na,1.7 pore-forming sequence alignments is in Table S1.
Electrophysiology experiments were performed on CHO cells transfected with an expression vector containing the full-length cDNA coding for the appropriate WT or mutant Na\textsubscript{a} sodium channel \(\alpha\)-subunit. The preparation of plasmids containing cDNA encoding for WT rNa\textsubscript{a}1.4 and hNa\textsubscript{a}1.7 has been described previously (41, 42). Cells were transfected using the molecular context for understanding differences in toxin affinities between different WT and mutant Na\textsubscript{a} isoforms. Our findings offer the rather exciting possibility that selective inhibitors of hNa\textsubscript{a}1.7 could be designed around site 1 because of the uniqueness of its outer pore structure vis-à-vis other sodium channel isoforms. In the absence of crystallographic data for mammalian Na\textsubscript{a}s, the rational design of Na\textsubscript{a}1.7 pore blockers should be enabled with our homology model.

**Materials and Methods**

**Electrophysiology.** Electrophysiology experiments were performed on CHO cells transfected with an expression vector containing the full-length cDNA coding for the appropriate WT or mutant Na\textsubscript{a} sodium channel \(\alpha\)-subunit. The preparation of plasmids containing cDNA encoding for WT rNa\textsubscript{a}1.4 and hNa\textsubscript{a}1.7 has been described previously (41, 42). Cells were transfected using

**Discussion**

Ion conduction in six of nine mammalian isoforms of Na\textsubscript{a} is blocked by TTX at low nanomolar concentrations (i.e., TTX-sensitive). The three so-called TTX-resistant isoforms differ by a 1-aa substitution in site 1, a structural modification that is responsible for the dramatic reduction in TTX affinity as shown by preparation of reciprocal mutants (32–34). Additionally, organisms that accumulate TTX or STX have evolved similar 1-aa variations in the outer vestibule of the channel that decrease toxin affinity—in some cases, by more than 1,000-fold (24, 35, 36). Studies that compare TTX and STX block of single-channel WT or mutant isoforms reveal parallel trends between the toxins (27, 37). Previous investigations of Na\textsubscript{a}1.7 from rabbit (38), cow (39), and rat (40) have shown that this isoform is both TTX- and STX-sensitive. In contrast to these reports, we have found that a naturally occurring double-point variation of two repeat III residues in hNa\textsubscript{a}1.7 results in selective destabilization of STX and GTX-III, but it is minimally perturbing on TTX binding. Intriguingly, this natural variation of adjacent amino acids in repeat III appears to be unique to primate Na\textsubscript{a}1.7 (Table 2 and Table S1).

The observed trends in toxin binding affinities noted in this study can be rationalized by differences in electrostatic and steric properties between the three toxins and six protein models. The rNa\textsubscript{a}1.4 D1241I mutant, for example, removes a negative charge from the putative toxin binding site. The binding of STX, which has the most positive surface electrostatic potential and thus would form the strongest electrostatic interaction with the native channel, is reduced by 19-fold to this mutant. Additionally, the rNa\textsubscript{a}1.4 M1240T mutation increases the volume of the binding site and reduces shape complementarity to the bound toxins. IC\textsubscript{50} values for STX and TTX binding are both decreased 26-fold with the inclusion of this mutation, whereas binding of the larger GTX-III to the more sterically open site is reduced by only 15-fold. In comparing toxin block of the single and double mutants of rNa\textsubscript{a}1.4, the effects of both amino acid changes seem to be additive: all three toxins exhibit decreased affinity to rNa\textsubscript{a}1.4 M1240T—D1241I. A similar rationale can be used to explain toxin binding affinity to the double mutant hNa\textsubscript{a}1.7 T1398M—I1399D compared with hNa\textsubscript{a}1.7.

We have identified a naturally occurring variation in 2 aa that line the outer pore region of Na\textsubscript{a} and significantly alters guanidinium toxin binding profiles. These findings refute conventional wisdom, which regards Na\textsubscript{a}1.7 as both a TTX- and STX-sensitive channel isoform. Homology modeling of the pore helix, P-loop, and S6 regions of the channel along with ligand docking studies provide a molecular context for understanding differences in toxin affinities between different WT and mutant Na\textsubscript{a} isoforms. Our findings offer the rather exciting possibility that selective inhibitors of hNa\textsubscript{a}1.7 could be designed around site 1 because of the uniqueness of its outer pore structure vis-à-vis other sodium channel isoforms. In the absence of crystallographic data for mammalian Na\textsubscript{a}s, the rational design of Na\textsubscript{a}1.7 pore blockers should be enabled with our homology model.

**Fig. 2.** Homology model-derived images highlighting differences between rNa\textsubscript{a}1.4 and hNa\textsubscript{a}1.7. STX bound in site 1 of the \(\alpha\)-subunit of the homology model of rNa\textsubscript{a}1.4 is viewed from (A) above and (B) the side of the pore. The four domains of the pore shown in cartoon representations and colored green (domain I), cyan (domain II), magenta (domain III), and yellow (domain IV). In B, residues comprising the DEKA selectivity filter are displayed as space-filling models; atoms are colored red (oxygen), blue (nitrogen), white (hydrogen), yellow (sulfur), and green (STX carbons) or white (protein carbons). In C–H, STX is docked into rNa\textsubscript{a}1.4 (C) and the identical pose in hNa\textsubscript{a}1.7 (D) to highlight the steric differences of the M1240T and D1241I variations. In C and D, STX is colored similarly as in A and B. The molecular surface of the protein is in white, with M1240 (C) or T1398 (D) shown in yellow and D1241 (C) or I1399 (D) in red. Electrostatic potential surfaces of rNa\textsubscript{a}1.4 (E), hNa\textsubscript{a}1.7 T1398M—I1399D (F), rNa\textsubscript{a}1.4 M1240T—D1241I (G), hNa\textsubscript{a}1.7 (H); depicted range is from −20 (red) to +5 kT (blue). In each image, residues 1,240 (1,398) and 1,241 (1,399) are displayed as stick figures.

**Fig. 3.** Electrostatic potential surfaces from −4 (red) to 4 kT (blue) of the toxins (A) TTX, (B) STX, and (C) GTX-III highlighting the differences in charge distributions between the molecules.
the method of calcium phosphate precipitation; cotransfection with eGFP was used as a marker of transfection efficiency.

Sodium currents were measured using the patch-clamp technique in the whole-cell configuration with an Axopatch-200b amplifier (Axon Instruments), which was previously described in the work by Moran et al. (43). Borosilicate glass micropipettes (Sutter Instruments) were fire-polished to a tip diameter yielding a resistance of 1.0–2.0 MΩ in the working solutions. The pipette was filled with 40 mM NaF, 1 mM EDTA, 20 mM Heps, and 125 mM CsCl, and the pH was adjusted to 7.4 with solid CsOH. The external solution had the following composition: 160 mM NaCl, 2 mM CaCl₂, and 20 mM Heps; the pH was adjusted to 7.4 with solid CsOH. Current densities were generally between 2 and 4 nA, except for the Na,1.4 M1240T mutant, which consistently gave higher current densities.

Stock solutions of each of the toxin derivatives (160 mM NaCl, 2 mM CaCl₂, 20 mM Heps; pH adjusted to 7.4 with solid CsOH) were maintained at 4 °C and diluted with external solution before recording. STX and GTX-III were purified and diluted with external solution before recording. STX, TTX, and GTX-III are considered to be mediators of local anesthetic action. STX, TTX, and GTX-III were performed using APBS (47). Specific details of the homology model creation and computational docking can be found in SI Materials and Methods. The homology models generated in this work are provided in Datasets S1, S2, S3, S4, S5, and S6.

Molecular Modeling. Homology models were created using Modeler (46). Computational ligand preparation was achieved using OpenEye tools Omega and Molcharge (OpenEye Scientific). Docked poses were initially generated using FRED (OpenEye Scientific) and minimized in LigandScout (InteLigand; Maria Enzersdorf Austria) under a built-in Merck Molecular Force Field. Surflex (Tripos) was used to relax the residues in the binding pocket to the minimized poses of the ligands. Electrostatic potential surface calculations were performed using APBS (47). Specific details of the homology model creation and computational docking can be found in SI Materials and Methods. The homology models generated in this work are provided in Datasets S1, S2, S3, S4, S5, and S6.

ACKNOWLEDGMENTS. J.R.W. and P.A.N. are research fellows of the Center for Molecular Analysis and Design (CMAD). W.H.P. is a recipient of a Stanford Interdisciplinary Graduate Fellowship (SIGF). Support from National Institutes of Health Grants R01-GM062888 (to V.S.P.), R01-N545684 (to J.D.B.), and R21-N5070064 (to J.D.B.) is gratefully acknowledged.