

Evaluation of toxicity equivalent factors of paralytic shellfish poisoning toxins in seven human sodium channels types by an automated high throughput electrophysiology system

Eva Alonso · Amparo Alfonso · Mercedes R. Vieytes ·
Luis M. Botana

Received: 20 October 2014 / Accepted: 17 December 2014 / Published online: 9 January 2015
© Springer-Verlag Berlin Heidelberg 2014

Abstract Although voltage-gated sodium channels (Na_v) are the cellular target of paralytic shellfish poisoning (PSP) toxins and that patch clamp electrophysiology is the most effective way of studying direct interaction of molecules with these channels, nowadays, this technique is still reduced to more specific analysis due to the difficulties of transforming it in a reliable throughput system. Actual functional methods for PSP detection are based in binding assays using receptors but not functional Na_v channels. Currently, the availability of automated patch clamp platforms and also of stably transfected cell lines with human Na_v channels allow us to introduce this specific and selective method for fast screenings in marine toxin detection. Taking advantage of the accessibility to pure PSP standards, we calculated the toxicity equivalent factors (TEFs) for nine PSP analogs obtaining reliable TEFs in human targets to fulfill the deficiencies of the official analytic methods and to verify automated patch clamp technology as a fast and reliable screening method for marine toxins that interact with the sodium channel. The main observation of this work was the large variation of TEFs depending on the channel subtype selected, being remarkable the variation of potency in the 1.7 channel subtype and the suitability of Na_v 1.6 and 1.2 channels for PSP screening.

Keywords Paralytic shellfish poisoning · Automated patch clamp · Sodium channels · Saxitoxin

E. Alonso · A. Alfonso · L. M. Botana (✉)
Departamento de Farmacología, Facultad de Veterinaria,
Universidad de Santiago de Compostela, 27002 Lugo, Spain
e-mail: Luis.Botana@usc.es

M. R. Vieytes
Departamento de Fisiología, Facultad de Veterinaria, Universidad
de Santiago de Compostela, 27003 Lugo, Spain

Introduction

Paralytic shellfish poisoning (PSP) is a serious food safety problem worldwide, triggered by the appearance of non-peptide neurotoxins produced by several species of *Alexandrium* genus and two specific dinoflagellates species, *Gymnodinium catenatum* and *Pyrodinium bahamense*. These compounds are accumulated in filter-feeding mollusks and located in their digestive tract, with the consequent risk of human consumption. This poisoning is characterized by neurological symptoms such as paralysis, dizziness, headache, and respiratory arrest, owing to the mechanism of action of this group of toxins, the blockage of sodium channels in excitable cells that interferes with the normal nervous transmission (Botana 2014; Lehane 2001).

PSP toxins are a group of more than 57 tetrahydropurines, closely related among them, and represented mainly by saxitoxin (STX; Wiese et al. 2010). STX basic structure is a 3,4-propinoperhydropurine which can be modified by addition of hydroxyl, carbamyl, *N*-sulfocarbamoyl, or sulfate groups (Fig. 1), producing several STX-analogues with different chemical properties and variations in potency. However, they share the same mechanism of action. Based on the relative potency of each STX-analogue, by means of their effect in sodium channels (Vale et al. 2008) and in the mouse bioassay (MBA), the European Food Safety Authority (EFSA) published the toxicity equivalency factors (TEF) of 15 PSP toxins, being the most potent NeOSTX, dcSTX, STX, and GTX1 (Fig. 1; EFSA 2009a). The legal limit for this group of toxins is 800 μg of STX equivalents/kg of meat or four mouse units/kg of meat, which corresponds to 18 μg of STX as the lethal amount needed for a 20 g mouse in 15 min (Vale 2014). For many years, the MBA has been the reference monitoring method for hydrophilic toxins and among them for PSP toxins detection (AOAC 1990). The

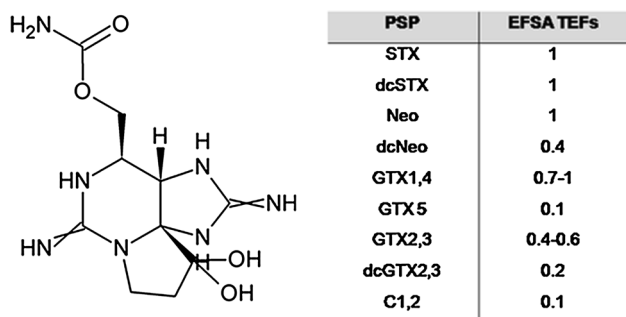


Fig. 1 Chemical structure of saxitoxin and relative potency of STX and analogues calculated by EFSA

MBA protocol was standardized and validated by inter-comparative studies; however, it is actually not considered as an appropriate tool due to the high variability between tests, the poor specificity and the limited detection provided (EFSA 2009b). Moreover, nowadays, the European legislation established that, following the experimental animal health protection, new methods are needed to better fit the “ThreeRs” rule (replacement, reduction, and refinement in experimental animal use; Hess et al. 2006). Based on this, different laboratories have developed analytical methods such as *in vitro* assays (cell receptor, enzymatic-based, and immunoassays; Fonfria et al. 2007; Fraga et al. 2012; van den Top et al. 2011; Velez et al. 2001) and/or chemical methods [high-performance liquid chromatography (HPLC) with ultraviolet, fluorescence, or mass spectrometry detection] (Ben-Gigirey et al. 2012). The European legislation allows the use of chromatographic analysis for saxitoxin and analogues (AOAC 2005). The use of analytical methods for PSP has been improved with a postcolumn method (AOAC 2011a), and the precolumn method has been extended (Turner and Hatfield 2012). The final interpretation of the values of these chromatographic analysis requires the conversion of each analog concentration to an equivalent amount of the reference compound, saxitoxin, by means of the use of the toxic equivalent factor or TEF (Botana et al. 2010). The use of TEF may be avoided by using functional assays that show the combined effect of the toxins on the targeted receptor, but up to now only one functional method based on a receptor binding assay has been approved by the AOAC as an official method (AOAC 2011b).

In this context, TEFs are essential to translate the analytical results obtained by chromatography methods into toxic values, since this technology provides a toxic profile of the sample but does not inform about its toxicity. As mentioned above, TEFs are calculated as the ratio of the relative toxicity of a PSP analog and the reference compound of the group, STX. Actually, in isomeric analogs, as gonyautoxin (GTX) 1,4 and GTX 2,3, the highest TEF is used, leading to an overestimation of the final toxicity in HPLC versus MBA.

Moreover, when no certified standard is attainable the reference compound is used instead and the same toxicity value is given to both compounds. Therefore, while analytical methods are the official standard procedure for PSP detection, the availability of pure and certified standards of each analog, as well as the access to reliable TEFs, is crucial.

The values previously obtained in our laboratory, by fluorescent and electrophysiological methods, of the blockade of sodium channels by PSP were the source for EFSA TEFs for PSP toxin screening (Perez et al. 2011; Vale et al. 2008). Since these data were obtained in primary neuronal cultures from mice, the availability of TEF values calculated in human Na_v channels would provide more accurate references to complete the routine analytical methods in human food security. Nonetheless, electrophysiological recordings assays, which has been essential for the advances in ion channel properties studies, are a harsh method that requires highly training operators and provides a low ratio of data generation, which turn patch clamp into a disadvantageous system for high throughput screenings. In the last years, different automated electrophysiology platforms have been released to the market to fulfill the pharmaceutical industry requirements with optimum results (Castle et al. 2009; Dunlop et al. 2008; Graef et al. 2013; Spencer et al. 2012).

The aim of this study was to obtain the toxicity equivalent factors of available PSPs toxins using an automated electrophysiology platform in different commercial available human Na_v transfected cell lines, on the basis of previously data obtained in our laboratory by manual patch clamp in mice neurons. This approach confirms the usefulness of this functional method to obtain reliable TEFs in human targets and provides a fast and reproducible method for high throughput screening.

Materials and methods

Chemicals and solutions

Plastic tissue cultures dishes were purchased from Falcon (Madrid, Spain). Fetal calf serum, Dulbecco's modified Eagle medium/F12 nutrient mixture (DMED/F12), glutamax, minimum essential medium, nonessential amino acids (MEM NEAA), and G418 were purchased from Gibco (Glasgow, UK). Detachin™ was purchased from Genlantis (USA).

The certified PSP standards were STX-dihydrochloride, dc-STX, Neo-STX, dcNeo-STX, GTX 1&4, dc-GTX 2&3 and GTX 5 were obtained from the Institute for Marine Biosciences, National Research Council of Canada, and GTX 2&3 and C1&2 were obtained from LaboratorioCifga, Spain. All other chemicals were of reagent grade and purchased from Sigma.

Stable mammalian cell line expressing hNa_v

HEK-293 cell line stably transfected with hNa_v 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, and 1.7 were kindly provided by Dr. Andrew Powell (GlaxoSmithKline R&D, Stevenage, UK). Cells were cultured in DMEM/F12 medium supplemented with glutamax, MEM NEAA (1 % w/v) and 10 % of fetal bovine serum. G418 was added at a final concentration of 0.4 mg/ml. Cells were incubated in a humidified 5 % CO₂/95 % air atmosphere at 37 °C until reach a 80 % of confluence. Then, cells are incubated at 30 °C for 24–48 h before electrophysiological measurements. Medium was replaced every 2–3 days and split once a week.

Automated Patch clamp electrophysiological recordings

All cells were recorded in whole-cell patch clamp configuration using an IonFlux 16 system (Fluxion, California, USA) and the corresponding Ionflux 16 software for cell capture, seal formation, whole cell obtaining, data acquisition and analysis. Cells maintained at 30 °C for 24–48 h were washed twice with Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) and harvested with 5 ml of Detachin™ solution. After cell detachment, cells were resuspended in extracellular solution containing (mM): 2 CaCl₂, 1 MgCl₂, 100 Hepes, 4 KCl, 145 NaCl, 10 TEA-Cl, and 10 glucose. pH 7.4 and 320 mOsm. Electrophysiological recordings were carried out at room temperature (±22 °C) in a 96-well Ion-Flux microfluidic plate. Briefly, Ionflux 16 is an automated patch clamp platform based in a microfluidic system where cells in suspension are captured by suction into ensemble recordings arrays formed by 20 individual microchannels for cell voltage clamp in parallel, each of which will trap one cell. Once that the recording assay is full, suction is applied to obtain the whole-cell configuration by cellular membrane breakage (Spencer et al. 2012).

For Na⁺ measurements in hNa_v channels, I_{Na} was evoked by depolarizing to –10 mV for 50 ms after a 100 ms step to –120 mV from –90 mV holding potential (V_h). The intracellular solution composition for I_{Na} recordings was (in mM): 100 CsF, 45 CsCl, 10 Hepes, five NaCl, five EGTA corrected to pH 7.1 using CsOH. The contaminating effects of resistance and capacitance currents were compensated electronically by the software. Leak resistance is measured by introducing a short 20 mV pulse at the beginning of each sweep and measuring the current difference. (Spencer et al. 2012). A sampling frequency of 10 kHz was used.

Toxicity equivalent factor estimation

TEF values for each PSP analog were obtained from IC₅₀ data as the ratio between the IC₅₀ obtained with parent compound STX and the IC₅₀ obtained with the analog.

Statistical analysis

Data analysis was performed using GraphPad Prism 5. All the assays were carried out at least three times. Dose-response curves were analyzed using nonlinear regression. All data are shown as the mean ± SEM.

Results

We had previously demonstrated that electrophysiological recordings provided a good correlation between *in vivo* PSPs potency and its *in vitro* sodium current inhibition in cerebellar neurons (Perez et al. 2011). In these neurons, Na_v 1.2 and Na_v 1.6 are the prevailing sodium subunits (Schaller and Caldwell 2003). The human Na_v 1.6 sodium channel is widely expressed in neurons and is the major voltage-gated sodium channels at nodes of Ranvier, but also in dendrites and synapses (Caldwell et al. 2000). Although it is also expressed in the peripheral nervous system, its larger presence in the central nervous system makes general classifications to include them among brain-type voltage-gated sodium channel. Na_v 1.2 is also a brain-type channel expressed in the central nervous system whose channelopathies produced an increased susceptibility to seizures (Savio-Galimberti et al. 2012). In this work, we want to reproduce the toxicity equivalent factor of available PSPs standards in a Na_v 1.6 and a Na_v 1.2 stably transfected cell line by electrophysiological recordings in an automated patch clamp platform to later compare it with different sodium channel types to find the most suitable for high throughput screenings and the more suitable TEFs for analytical data interpretation.

Firstly, we studied the effect of nine PSP standards over the voltage-dependent sodium currents (I_{Na}) in these two human sodium channel subunits, Na_v 1.6 and Na_v 1.2. For this, electrophysiological recordings were done in an automated patch clamp system to obtain the half maximal inhibitory concentration (IC₅₀). As shown in Fig. 2a, STX elicited a dose-dependent inhibition of I_{Na} with practically the same IC₅₀ for both subunits, 1.09 nM [95 % confidence interval (CI) 0.66–1.8 nM] for Na_v 1.6 and 1 nM (95 % CI 0.56–1.8 nM) for Na_v 1.2. Accordingly, in Na_v 1.6 cells dcSTX inhibited with a similar potency the sodium current with an IC₅₀ of 1.13 nM (95 % CI 0.55–1.3 nM). The NeoSTX standard showed a slightly higher potency with an IC₅₀ of 0.86 nM (95 % CI 0.63–1.1 nM), whereas the analog dcNeoSTX was the least powerful of this group yielding an IC₅₀ of 4.36 nM (95 % CI 2.54–7.46 nM). Likewise, these values were comparable with those obtained in Na_v 1.2, where NeoSTX was also the most powerful (0.39 nM, 95 % CI 0.28–0.53 nM), followed by dcSTX (4.09 nM, 95 % CI 2.36–6.79 nM) and finally again,

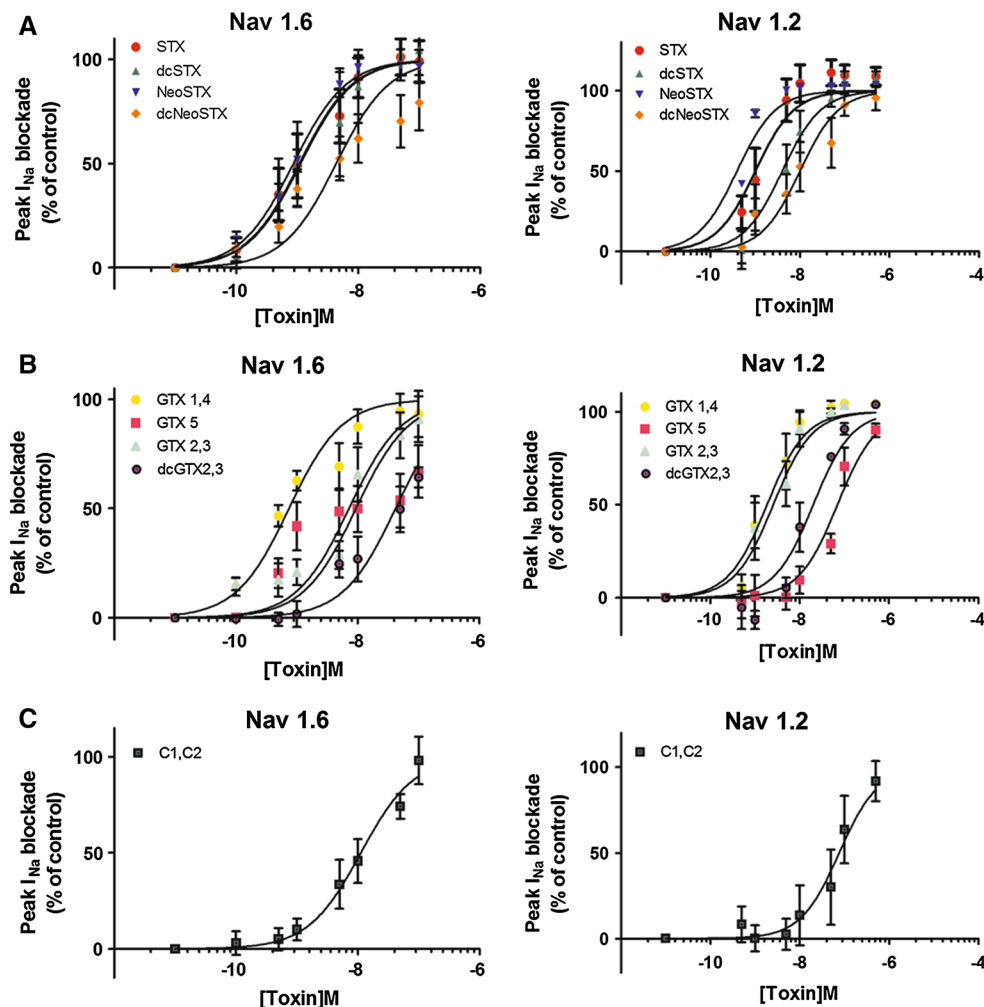


Fig. 2 Dose–response curves for PSP standards of **a** STX, dcSTX, NeoSTX, and dcNeo, **b** GTX 1,4, GTX 5, GTX 2,3, and dcGTX 2,3 and **c** C1,2 on voltage-dependent sodium currents expressed in

HEK293 hNa_v 1.2 and 1.6. Values are the mean \pm SEM of four independent experiments

dcNeoSTX with the highest IC₅₀ of the group, 9.9 nM (95 % CI 5.78–17.12 nM).

The GTX group was also evaluated (Fig. 2b), and as before, the IC₅₀ obtained for both subunits was near the same values. GTX 1,4 inhibited I_{Na} with an IC₅₀ of 0.76 nM (95 % CI 0.49–1.1 nM) and 1.8 nM (95 % CI 1.19–2.86 nM), respectively, for Na_v 1.6 and Na_v 1.2. Slightly higher values were obtained for GTX 2,3, 7.2 nM (95 % CI 4.5–11.3 nM) and 2.55 nM (95 % CI 1.5–4.35 nM), respectively, and a less powerful inhibition was seen in the case of dcGTX 2,3, with 41.8 nM (95 % CI 29.2–59.8 nM) for Na_v 1.6 and 20.05 nM (95 % CI 13.46–29.88 nM) for Na_v 1.2. However, the values calculated for GTX 5 were remarkable different for both subunits. While the IC₅₀ for Na_v 1.6 was 9.41 nM (95 % CI 5.01–17.7 nM), this toxin blocked Na_v 1.2 currents with an IC₅₀ seven times higher than the observed in Na_v 1.6, 69.8 nM (95 % CI 46.84–104 nM).

Likewise, the C1,2 standard also displayed a significant variation between these two subunits. The value for Na_v 1.6 was again almost seven times lower than the one for Na_v 1.2, 11.15 nM (95 % CI 7.45–16.7 nM), and 76.15 nM (95 % CI 39.42–147.1 nM; Fig. 2c).

With these estimations the TEFs for both Na_v subunits were calculated and compared with the values proposed by EFSA, which are a combination of TEFs obtained in cerebellar neurons (Vale et al. 2008) and MBA (Oshima 1995; Fig. 3), and the recently calculated TEFs for several PSP standards with the acute toxicities after oral administrations in mice (Munday et al. 2013). Even though for almost all the PSP standards the values were similar, the relative potencies obtained with Na_v 1.6 cells were closer to the values previously determined in animals, specially to those previously obtained in cerebellar neurons, which constitute the main source of EFSA TEFs

Fig. 3 Linear relationship between the in vitro potency of PSP standards obtained in HEK293 hNa_v 1.6 cells (a) or in hNa_v 1.2 (b) versus the toxicity equivalent factors for PSP published by EFSA. c Comparative results of the toxicity equivalent factors calculated by EFSA, the observed after oral administration and the obtained in cerebellar neurons in manual patch clamp versus the obtained in HEK293 Na_v 1.6 and 1.2 in automated patch clamp

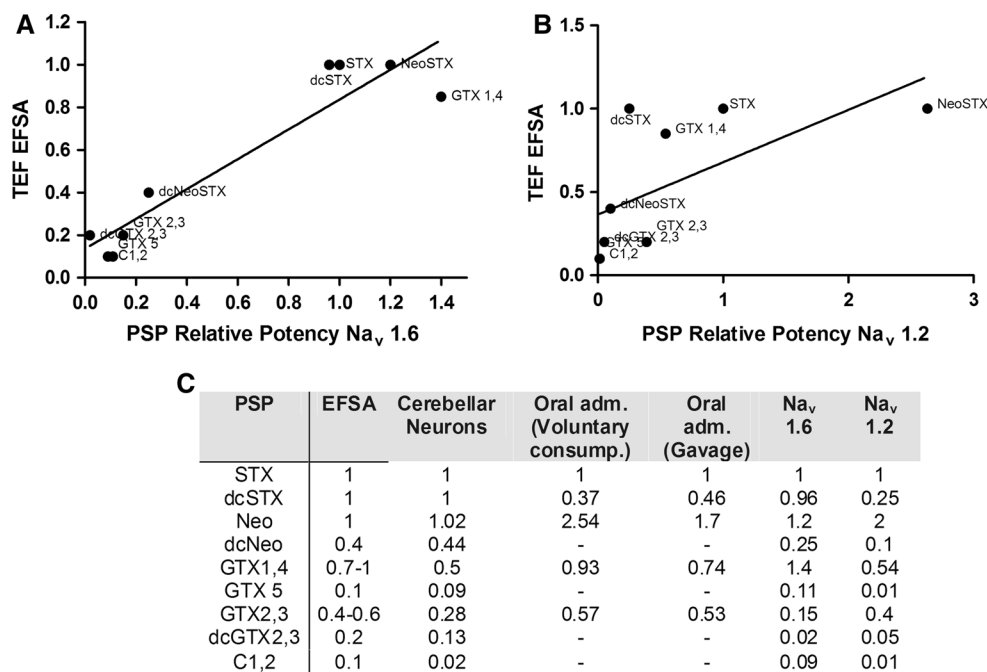
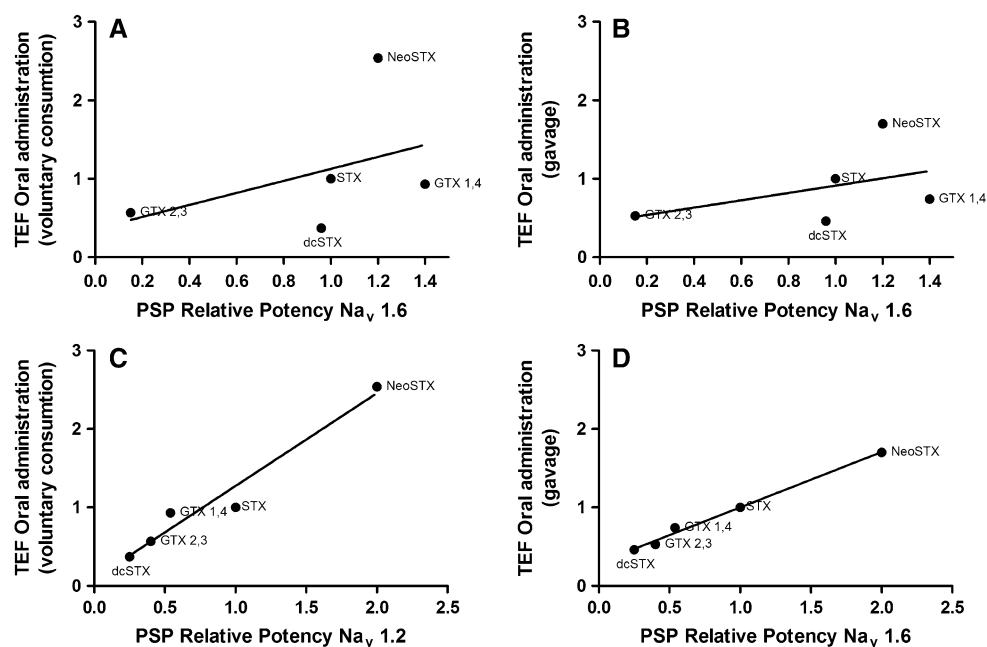


Fig. 4 Linear relationship between the in vitro potency of PSP standards obtained in HEK293 hNa_v 1.6 cells (a, b) or in hNa_v 1.2 (b, c) versus data obtained in oral administrations



(Fig. 3c). These data indicate that NeoSTX and GTX1,4 have a higher toxicity risk than STX and dcSTX, which support previous data after oral administration (Munday et al. 2013) showing that the GTX1,4 mixture could have a larger TEF value than the assigned by EFSA. However, if we only focused in the data obtained with the four PSP toxins used in oral administration by voluntary consumption and gavage in mouse, we can observe that the linear correlation in both assumptions had a better correspondence with Na_v 1.2 data (Fig. 4). This is due to the high

oral toxicity of NeoSTX, especially observed in the voluntary consumption, and the lower values of dcSTX versus the showed ones in Na_v 1.6. Therefore, it can be concluded that the values obtained in the automated patch clamp platform in Na_v 1.6 transfected cells exhibited a great correlation with the values obtained in vivo and published by EFSA. However, a more exhaustive study with the nine PSP toxins used in the present work in oral administrations could point out a better suitability of the Na_v 1.2 subunit.

Table 1 IC₅₀ values and TEF for each Na_v channel

Na _v subunit PSP standard	Na _v 1.1	Na _v 1.2	Na _v 1.3	Na _v 1.4	Na _v 1.5	Na _v 1.6	Na _v 1.7
STX							
IC ₅₀	2.3	1	13.4	1.88	212.6	1.09	408
TEF	1	1	1	1	1	1	1
TEF _{Nav1.6}	0.47	1.09	0.008	0.58	0.005	1	0.002
dcSTX							
IC ₅₀	33.43	4.01	156.5	11.65	221.6	1.13	88.5
TEF	0.07	0.25	0.08	0.16	0.96	0.96	4.6
TEF _{Nav1.6}	0.03	0.27	0.007	0.09	0.005	0.96	0.01
Neo							
IC ₅₀	0.36	0.38	4.7	0.71	5.5	0.86	81.6
TEF	6.4	2.63	2.85	2.6	38.6	1.2	5
TEF _{Nav1.6}	3	2.8	0.23	1.5	0.2	1.2	0.01
dcNeo							
IC ₅₀	1,700	9.94	563	1,240	291.4	4.4	1,240
TEF	0.001	0.1	0.024	0.001	0.73	0.25	0.33
TEF _{Nav1.6}	0.0006	0.11	0.001	0.0008	0.003	0.25	0.0008
GTX1,4							
IC ₅₀	2.4	1.85	8.3	3.29	14.77	0.74	43.9
TEF	0.96	0.54	1.6	0.57	14.4	1.4	9.29
TEF _{Nav1.6}	0.45	0.59	0.13	0.33	0.07	1.4	0.002
GTX5							
IC ₅₀	147.2	69.8	155.9	10.13	19.92	9.4	100
TEF	0.015	0.014	0.08	0.18	10.7	0.11	4.08
TEF _{Nav1.6}	0.007	0.02	0.007	0.11	0.05	0.11	0.01
GTX2,3							
IC ₅₀	10.88	2.55	20.8	5.78	54.88	7.2	1,500
TEF	0.2	0.39	0.64	0.32	3.87	0.15	0.27
TEF _{Nav1.6}	0.1	0.43	0.05	0.19	0.02	0.5	0.0007
dcGTX2,3							
IC ₅₀	54.88	20.05	61.4	114.4	64.59	41.8	130
TEF	0.04	0.05	0.22	0.01	3.3	0.02	3.1
TEF _{Nav1.6}	0.02	0.05	0.018	0.009	0.017	0.02	0.008
C1,2							
IC ₅₀	271.8	76.15	53.2	2,093	82.1	11.5	3,700
TEF	0.008	0.013	0.25	ND	2.6	0.09	0.1
TEF _{Nav1.6}	0.004	0.02	0.02	0.0005	0.01	0.09	0.0002

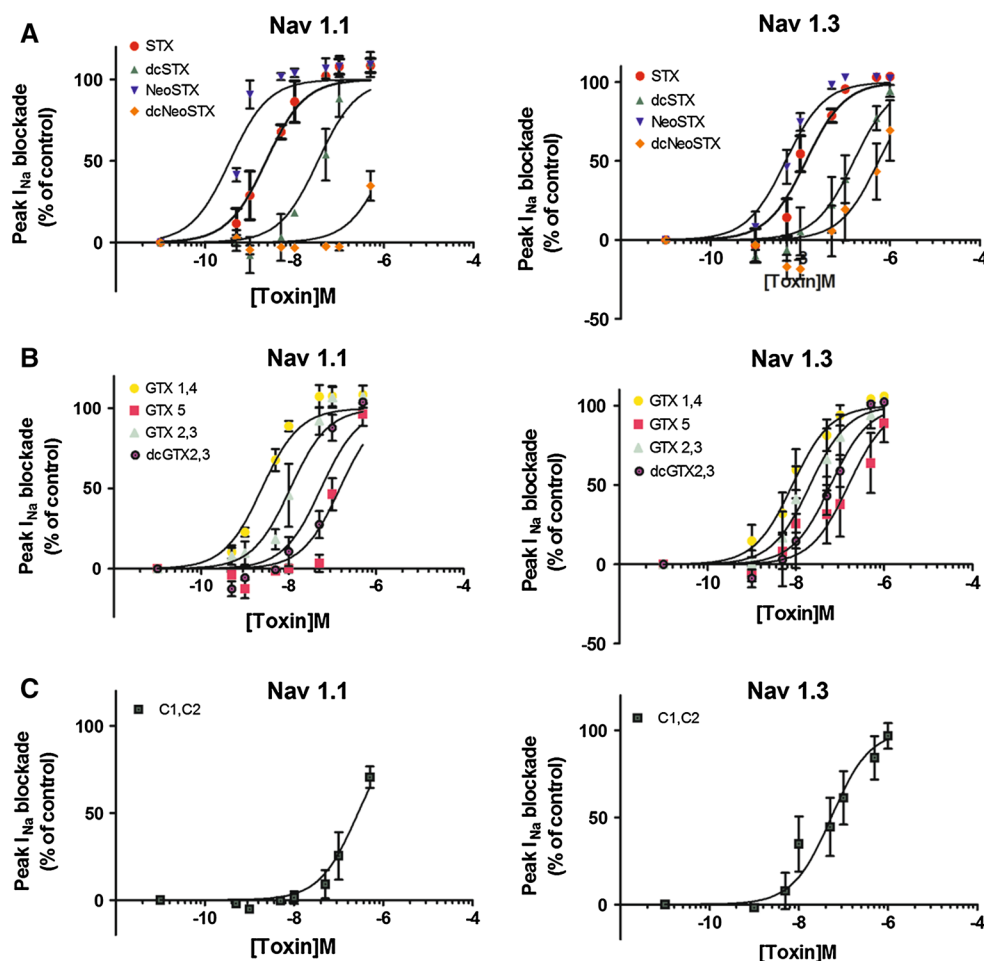
IC₅₀ values (nM, $n \geq 3$ cells) for nine PSP standards. TEF calculated for each PSP standard and each Na_v subunit with STX IC₅₀ value as the reference value for each subunit (TEF) or TEF calculated with STX IC₅₀ in Na_v 1.6 as the reference value for all the other subunits (TEF_{Nav1.6})

Among the 10 mammalian Na_v subunits, Na_v 1.2 and Na_v 1.6 belong to the neuronal or brain-type and are expressed mainly in the central nervous system; hence, we decided to perform a comparative study in different Na_v subunits expressed in various tissues. We studied four neuronal-type channels, the previously studied Na_v 1.2 and Na_v 1.6, and moreover the Na_v 1.1 and Na_v 1.3. The Na_v 1.4 (mainly presented in skeletal muscle), the Na_v 1.5 (the cardiac specific subunit) and an isoform predominant located in the peripheral nervous system, Na_v 1.7 (Savio-Galimberti et al. 2012). All of them were stably expressed in HEK-293 cells. In Table 1 and Figs. 4, 5 and 6, the

values of the nine PSP IC₅₀ standards for the seven Na_v subunits tested are summarized.

It is remarkable that in the neuronal-type subunits all the values are always lower than 157 nM, being the highest value the elicited by dcSTX in Na_v 1.3 currents (156.5 nM, 95 % CI 90.52–270.6 nM), but most of them are in the low nanomolar range (Fig. 5). However, the cardiac subunit, Na_v 1.5 that is also the only tetrodotoxin-resistant type analyzed (Fig. 6; Catterall et al. 2005), presented considerable higher IC₅₀ for STX, dcSTX and dcNeoSTX, all of them around 200 nM and only a low concentration of 5.5 nM (95 % CI 3.02–10.02 nM) for NeoSTX. Most surprising are

Fig. 5 Dose–response curves for PSP standards of **a** STX, dcSTX, NeoSTX, and dcNeo, **b** GTX 1,4, GTX 5, GTX 2,3, and dcGTX 2,3 and **c** C1,2 on voltage-dependent sodium currents elicited in the brain-type subunits hNa_v 1.1 and 1.3. Values are the mean ± SEM of four independent experiments



the data obtained with the GTX group in Na_v 1.5 (Fig. 6b), where GTX 1.4 has an IC₅₀ of 14.77 and GTX 5 of 19.92 nM (95 % CI 7.89–27.63 and 8.63–45.9 nM, respectively). Additionally, the other two GTX components, GTX 2,3 and dcGTX 2,3, showed IC₅₀ with concentrations below 70 nM that are still significantly lower than those observed by STX, dcSTX, and dcNeo.

The subunit expressed in the skeletal muscle, Na_v 1.4, provided instead the expected IC₅₀ values for PSP standards (Table 1; Fig. 6), where the parent compound elicited an inhibition of sodium currents with an IC₅₀ of 1.88 nM (95 % CI 1.1–3.20 nM; Fig. 6a), similar to the previously obtained 2.8 nM, calculated in the rat Na_v 1.4 expressed in CHO cells instead than in HEK cells as in the present work (Walker et al. 2012).

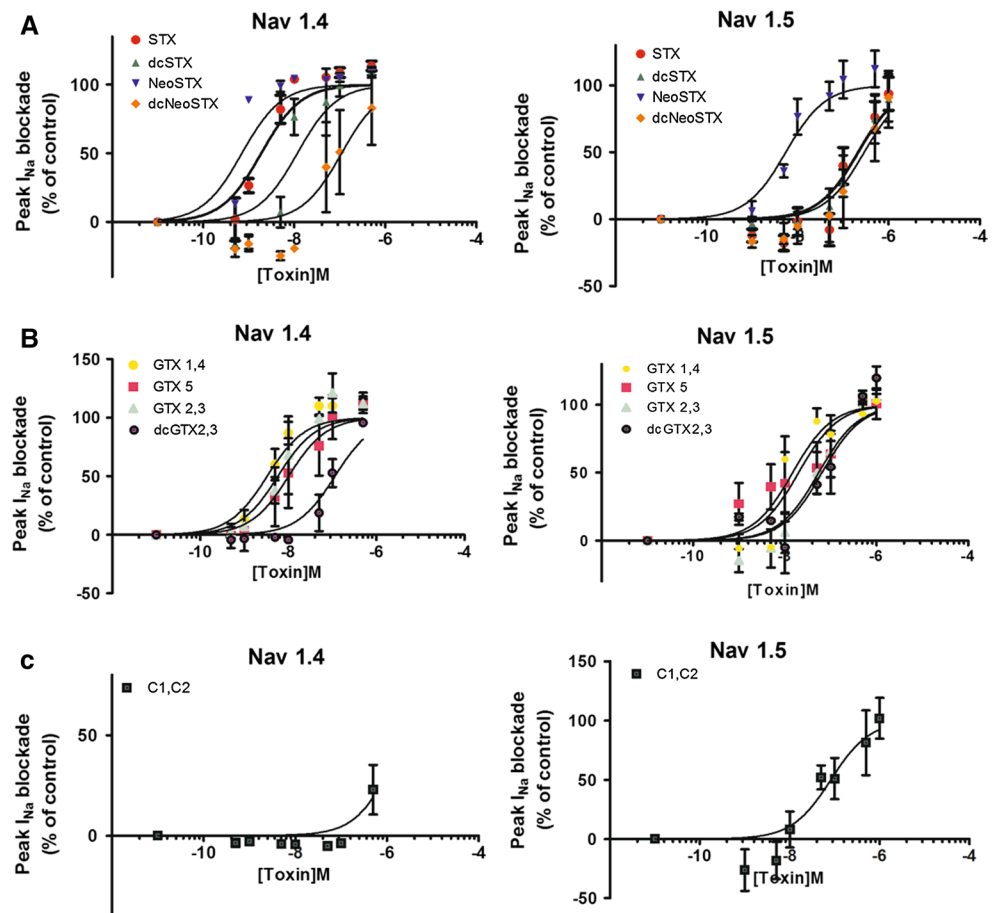
Finally, Na_v 1.7 was the only subunit mainly expressed in the peripheral nervous system of the seven subunits analyzed (Fig. 7). This TTX-sensitive subunit provided the highest IC₅₀ for all the PSP toxins tested. The parent compound, STX elicited an I_{Na} inhibition with an IC₅₀ of 408 nM (95 % CI 212.1–784.9 nM; Fig. 7). This value is 400 times higher than the one obtained with the other

TTX-sensitive subunits and even two times higher than that obtained with the TTX resistant subunit Na_v 1.5. These data are in agreement with previously reported values in this subunit in a comparative study between Na_v 1.7 and Na_v 1.4 and their, respectively, affinities for STX and TTX, where Na_v 1.7 showed a significant major response to TTX than to STX (Walker et al. 2012). Furthermore, the IC₅₀ obtained for dcNeo, GTX2,3, and C1,2 was all in the micromolar range for this specific subunit (Fig. 7), which clearly turn this Na_v 1.7 subunit in the less sensitive to the PSP toxins.

Discussion

Nowadays, with the change of the European legislation and the establishment of the chromatographic methods as substitutes for MBA in PSP detection, the use of reliable TEFs is a needed tool for food safety controls. TEFs can be calculated with the previous knowledge of in vivo toxicity (intraperitoneal administration in mice) or in vitro (with mice neuronal channels). Previously, based in the mechanistic action of these toxins, a good correlation between

Fig. 6 Dose–response curves for PSP standards of **a** STX, dcSTX, NeoSTX, and dcNeo, **b** GTX 1,4, GTX 5, GTX 2,3, and dcGTX 2,3 and **c** C1,2 on voltage-dependent sodium currents elicited in the skeletal muscle-type subunit hNa_v 1.4 and the cardiac-type subunit hNa_v 1.5. Values are the mean \pm SEM of four independent experiments



in vivo and in vitro TEFs was calculated by electrophysiological measurements in cerebellar neurons in our laboratory (Perez et al. 2011). However, electrophysiological techniques require expert technicians and a considerable time to evaluate huge amount of samples. In the present work, we present the TEFs calculated for nine PSPs toxin standards in seven different human Na_v subunits using an automated patch clamp platform which a 96-well system that allow the simultaneous analysis of different samples. Moreover, the use of HEK293 cells stable transfected with different Na_v subunits permit us to study the specific effect of each standard over the different subunits in order to find the most accurate one to the in vivo data. The results summarized in the present work allow us to settle that the TEFs obtained with the Na_v 1.6 subunit possess the strongest equivalence with the MBA and those previously obtained in cerebellar neurons, being the relative potency of PSP standards GTX1,4 \approx NeoSTX \approx STX > dcSTX > dcNeo > GTX 2,3 \approx GTX5 > C1,2 > dcGTX2,3 (Fig. 3). However, the Na_v 1.2 subunit seems to have a better correlation with the data obtained after oral administration in mouse, but a larger study with more compounds would be necessary to assert which of both subunits has a better correspondence in this case. The Na_v 1.6 channel has been widely studied and its

biophysical characterization proved that it produced a transient and persistent inward sodium current. Na_v 1.6 channel has a key role in the action potential transmission and mice carrying null Na_v 1.6 mutations suffering from cerebellar ataxia (Kohrman et al. 1996). Despite that the IC₅₀ obtained by PSP toxins for all the brain-type subunits (Na_v 1.1, 1.2, 1.3 and 1.6) is all in the low nanomolar range, it is Na_v 1.6 which has the highest parallelism in relative potency with the acute administration in mice. Therefore, this Na_v subunit has proved to be a good tool for PSP screening using the own target of this group of marine toxins, the sodium channel, and using a very sensitive technique as patch clamp but without the drawbacks of this assay. The automated platform permits a faster and easier training than manual patch clamp and, moreover, the simultaneous analysis of different samples. Likewise, the usage of a “ready to use” transfected cell line is in accordance with the European legislation in experimental animal protection, since these transfected cells express great sodium currents with noninterference of other channels and fulfilling the refinement, reduction, and replacement of animals. Moreover, the use of a human cell line and human Na_v channel subunits provide us with more accurate data for human security, avoiding the possible interferences between species and strains.

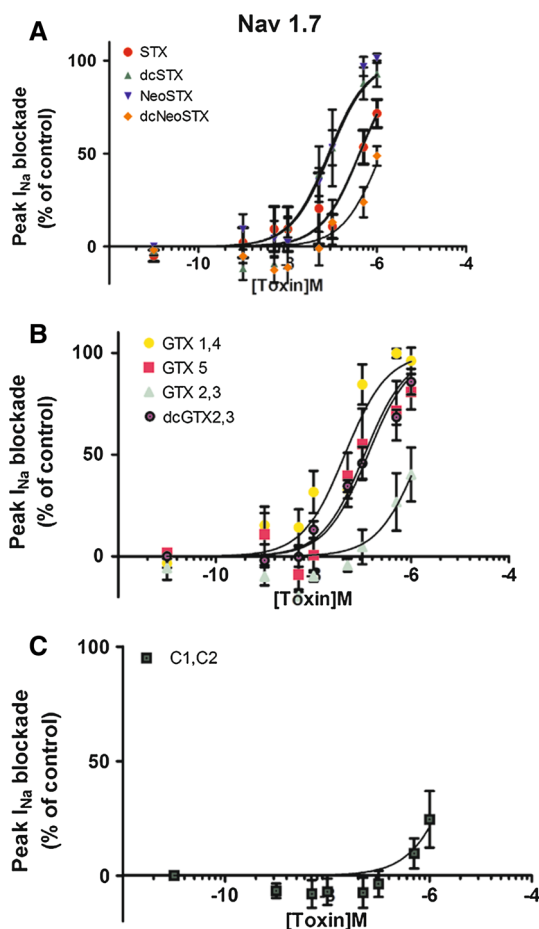


Fig. 7 Dose–response curves for PSP standards of **a** STX, dcSTX, NeoSTX, and dcNeo, **b** GTX 1,4, GTX 5, GTX 2,3, and dcGTX 2,3 and **c** C1,2 on voltage-dependent sodium currents observed in the peripheral nervous system subunit hNa_v 1.7 expressed in HEK293 cells. Values are the mean \pm SEM of four independent experiments

Among the seven human Na_v subunits analyzed only one tetrodotoxin-resistant was tested, the Na_v 1.5. However, it is reasonable to think that due to the fact that the three resistant isoforms, Na_v 1.5, 1.8, and 1.9, share the same structural modification in site 1 that is responsible for the lower affinity to tetrodotoxin and PSPs (Leffler et al. 2005; Sivillotti et al. 1997), similar data would be obtained in Na_v 1.8 and 1.9 that will discard these subunits as the more accurate ones at least for PSP measurement. Furthermore, the tetrodotoxin-sensitive Na_v 1.7 is the less affected by PSP toxins in this study, with IC₅₀ values markedly higher than the ones obtained in the other subunits. While the relative potency relationship of the standards follows a common pattern in most of the sodium subunits, where the most potent compounds are Neo-STX, GTX 1,4 or the parent compound STX, only in the cardiac Na_v 1.5 and the peripheral Na_v 1.7 this criterion is disturbed and even though NeoSTX and GTX 1,4 still appear as the most

potent standards the parent compound falls to the 5th and 7th position in Na_v 1.7 and Na_v 1.5, respectively. In this sense, it has been recently proved that the Na_v 1.7 subunit possesses two aminoacid sequence modifications in binding site 1 compared with the other tetrodotoxin-sensitive subunits that justify its lower binding affinities for STX and GTX-3. This mutation appears as primate-specific causing marked differences with the affinities of other mammals Na_v 1.7 subunits (Walker et al. 2012).

Thus, the method described in the present work settled that automated patch clamp with the commercial available transfected human Na_v 1.6 cell line represents a great instrument to combine with the analytical methods and therefore to translate these analytic results into toxic values using small amounts of standards and with certainly low IC₅₀ values with a high throughput compatible system. The fact that the transfected model uses human cells brings the use of these TEF closer to human food safety needs.

A summary of all results is presented in Table 1, where the TEF for each channel subtype is shown along with the corresponding TEF referred to the 1.6 subtype, which provides the closest value to the animal and in vitro derived TEF proposed by EFSA. The analysis of these results suggests that the selection of different channel subtypes for an in vitro test would provide totally different results. Also, the density of receptor subtypes in the tissues of a given strain of animals may explain the differences observed sometimes.

Acknowledgments The research leading to these results has received funding from the following FEDER cofounded-grants. From CDTI and Technological Funds, supported by Ministerio de Economía y Competitividad, AGL2012-40185-CO2-01 and Consellería de Cultura, Educación e Ordenación Universitaria, GRC2013-016, and through Axencia Galega de Innovación, Spain, ITC-20133020 SINTOX, IN852A 2013/16-3 MYTIGAL. From CDTI under ISIP Programme, Spain, IDI-20130304 APTAFOOD. From the European Union's Seventh Framework Programme managed by REA—Research Executive Agency (FP7/2007–2013) under Grant Agreement Nos. 265409 μ AQUA, 315285 CIGUATOOLS, and 312184 PHARMASEA.

Conflict of interest The authors declare that they have no conflict of interest.

References

- AOAC (1990) Paralytic shellfish poison. Biological method. Final action. In: Hellrich EBK (ed) Official methods of analysis. Association of Official Methods of Analytical Chemists, Arlington, VA, pp 881–882
- AOAC (2005) Method 2005.06: Paralytic shellfish poisoning toxins in shellfish. Prechromatographic oxidation and liquid chromatography with fluorescence detection. Official Methods of Analysis of the Association of Official Analytical Chemists., Method 2005.06, First Action.) (Commission, E. (2006). Commission

- Regulation (EC) No 1664/2006 of 6 November 2006 amending Regulation (EC) No 2074/2005 as regards implementing measures for certain products of animal origin intended for human consumption and repealing certain implementing measures. *Off J Eur Union L* 320:13–45
- AOAC (2011a) AOAC Official method 2011.02 Determination of paralytic shellfish poisoning toxins in mussels, clams, oysters and scallops. Post-column oxidation method (PCOX). First action 2011. In *MD Official Methods of Analysis of the AOAC* (Association of Official Analytical Chemists) Gaithersburg, USA, Method 2011.02
- AOAC (2011b) AOAC official method 2011.27. In *Paralytic shellfish toxins (PSTs) in shellfish, receptor binding assay*. Association of Official Methods of Analytical Chemists, Arlington, VA; AOAC International: AOAC International, Gaithersburg, MD), pp 881–882
- Ben-Gigirey B et al (2012) A comparative study for PSP toxins quantification by using MBA and HPLC official methods in shellfish. *Toxicon* 60(5):864–873
- Botana LM (ed) (2014) *Seafood and freshwater toxins. Pharmacology, physiology and detection*, 3rd ed. CRC Press, Boca Raton
- Botana LM, Vilarinho N, Elliott CT, Campbell K, Alfonso A, Vale C, Louzao MC, Botana AM (2010) ‘The problem of toxicity equivalent factors in developing alternative methods to animal bioassays for marine toxin detection. *Trends Anal Chem* 29:1316–1325
- Caldwell JH et al (2000) Sodium channel Na(v)1.6 is localized at nodes of ranvier, dendrites, and synapses. *Proc Natl Acad Sci USA* 97(10):5616–5620
- Castle N et al (2009) Sodium channel inhibitor drug discovery using automated high throughput electrophysiology platforms. *Comb Chem High Throughput Screen* 12(1):107–122
- Catterall WA, Goldin AL, Waxman SG (2005) International Union of Pharmacology. XLVII. Nomenclature and structure–function relationships of voltage-gated sodium channels. *Pharmacol Rev* 57(4):397–409
- Dunlop J et al (2008) High-throughput electrophysiology: an emerging paradigm for ion-channel screening and physiology. *Nat Rev Drug Discov* 7(4):358–368
- EFSA (2009a) Scientific opinion of the panel on contaminants in the food chain: a request from the European Commission on marine biotoxins in shellfish: saxitoxin group (question no EFSA-Q-2006-065E). *EFSA J* 1019:1–76
- EFSA (2009b) Scientific opinion on marine biotoxins in shellfish—summary on regulated marine biotoxins. *EFSA J* 1306:1–23
- Fonfria ES et al (2007) Paralytic shellfish poisoning detection by surface plasmon resonance-based biosensors in shellfish matrices. *Anal Chem* 79(16):6303–6311
- Fraga M et al (2012) Detection of paralytic shellfish toxins by a solid-phase inhibition immunoassay using a microsphere-flow cytometry system. *Anal Chem* 84(10):4350–4356
- Graef JD et al (2013) Validation of a high-throughput, automated electrophysiology platform for the screening of nicotinic agonists and antagonists. *J Biomol Screen* 18(1):116–127
- Hess P et al (2006) Three Rs Approaches in marine biotoxin testing. The Report and Recommendations of a joint ECVAM/DG SANCO workshop (ECVAM workshop 54). *Altern Lab Anim* 34(2):193–224
- Kohrman DC et al (1996) A missense mutation in the sodium channel Scn8a is responsible for cerebellar ataxia in the mouse mutant jolting. *J Neurosci* 16(19):5993–5999
- Leffler A et al (2005) Pharmacological properties of neuronal TTX-resistant sodium channels and the role of a critical serine pore residue. *Pflugers Arch* 451(3):454–463
- Lehane L (2001) Paralytic shellfish poisoning: a potential public health problem. *Med J Aust* 175(1):29–31
- Munday R et al (2013) Acute toxicities of saxitoxin, neosaxitoxin, decarbamoyl saxitoxin and gonyautoxins 1&4 and 2&3 to mice by various routes of administration. *Toxicon* 76:77–83
- Oshima Y (1995) Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. *J AOAC Int* 78:528–532
- Perez S et al (2011) Determination of toxicity equivalent factors for paralytic shellfish toxins by electrophysiological measurements in cultured neurons. *Chem Res Toxicol* 24(7):1153–1157
- Savio-Galimberti E, Gollob MH, Darbar D (2012) Voltage-gated sodium channels: biophysics, pharmacology, and related channelopathies. *Front Pharmacol* 3:124
- Schaller KL, Caldwell JH (2003) Expression and distribution of voltage-gated sodium channels in the cerebellum. *Cerebellum* 2(1):2–9
- Sivilotti L et al (1997) A single serine residue confers tetrodotoxin insensitivity on the rat sensory-neuron-specific sodium channel SNS. *FEBS Lett* 409(1):49–52
- Spencer CI et al (2012) Ion channel pharmacology under flow: automation via well-plate microfluidics. *Assay Drug Dev Technol* 10(4):313–324
- Turner AD, Hatfield RG (2012) Refinement of AOAC official method 2005.06 liquid chromatography–fluorescence detection method to improve performance characteristics for the determination of paralytic shellfish toxins in king and queen scallops. *J AOAC Int* 95:129–142
- Vale P (2014) Saxitoxin and analogs: ecobiology, origin, chemistry and detection. In: Botana LM (ed) *Seafood and freshwater toxins. Pharmacology, physiology and detection*, 3rd edn. CRC Press, Boca Raton
- Vale C et al (2008) In vitro and in vivo evaluation of paralytic shellfish poisoning toxin potency and the influence of the pH of extraction. *Anal Chem* 80(5):1770–1776
- van den Top HJ et al (2011) Surface plasmon resonance biosensor screening method for paralytic shellfish poisoning toxins: a pilot interlaboratory study. *Anal Chem* 83(11):4206–4213
- Velez P et al (2001) A functional assay for paralytic shellfish toxins that uses recombinant sodium channels. *Toxicon* 39(7):929–935
- Walker JR et al (2012) Marked difference in saxitoxin and tetrodotoxin affinity for the human nociceptive voltage-gated sodium channel (Na_v 1.7) [corrected]. *Proc Natl Acad Sci U S A* 109(44):18102–18107
- Wiese M et al (2010) Neurotoxic alkaloids: saxitoxin and its analogs. *Mar Drugs* 8(7):2185–2211