The Effect of the Cyanobacterial Toxin Saxitoxin on Neurodevelopment

By

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Abstract

The potent neurotoxin saxitoxin (STX) belongs to a group of structurally related analogues, collectively known as the paralytic shellfish toxins, produced by both marine and freshwater phytoplankton. This group of toxins act by blocking the voltage-gated sodium channels, halting the inflow of sodium ions and the subsequent generation of action potentials. While acute exposure has been well researched, with safety guidelines applied, chronic low dose exposure from neither marine or freshwater sources has been investigated. Given the role of cellular electrical activity in neurodevelopment this latter pattern of exposure may be of significant public health concern. This background has been addressed in chapter 1; “Low dose extended exposure to saxitoxin and its potential neurodevelopmental effects: a review”, and the published manuscript can be found in Appendix 1.

Given this lack of investigation we aimed to determine if STX had an adverse effect on neurodevelopment following low dose extended exposure using two models of neuronal development. Further, we aimed to establish an assay which could be used to determine if any adverse neurodevelopmental effects recorded were due to direct STX toxicity.

Firstly, using model neuronal cell lines it was shown that STX at or below the current drinking water guideline (0.25-3μg/L) caused a significant concentration dependent decrease in the development of neuronal morphology following an extended exposure period. This research is presented in chapter 2; “Extended low-dose exposure to saxitoxin inhibits

neurite outgrowth in model neuronal cells" and the published manuscript can be found in Appendix 2.

In addition to investigating the neurodevelopmental effects of STX, an assay measuring viability indirectly through cellular metabolism was established to be used with STX. The assay was used to eliminate the possibility of non-specific cell toxicity as a cause of the effects on neurodevelopment. The assay was successfully optimised in two cell lines and tested with STX (0.25-10μg/L) and $ZnSO_4$ (10^{-4}-10^{-1}M), a known cytotoxic compound. The assay showed that STX is not toxic in our cell line under the conditions used for chapter 2. These results are reported in Chapter 3; "Optimisation of a real time resazurin based assay for use in OVCAR-3 and SH-SY5Y cells".

Moving to a model which more accurately models mammalian neuronal differentiation, the effect of STX at the drinking water guideline (3μg/L) and a predicted algal bloom concentration (10μg/L) was investigated using embryonic stem cells. Cells were differentiated using a previously described method of neuronal differentiation and assessed by examination of morphological development of neuronal features and expression of gene markers. A concentration dependent decrease in morphological neuronal index scores was recorded, confirming the results of chapter 1, in addition the expression of neuronal markers *nestin* and *MAP2* were increased following exposure to STX (3μg/L) while *β_{III}-Tubulin* was delayed by 3 days in both STX treatment groups. This research is presented in chapter 4; "Low dose exposure to saxitoxin affects neuronal differentiation of D3 embryonic stem cells".

These results suggest that STX, and potentially its analogues, interfere with proper neuronal development at environmentally relevant concentrations. Whilst further work is required to investigate the mechanisms causing the adverse effects seen, the work presented here raises awareness that this pattern of exposure could be of significant public health concern and deserves further investigation.

Student declaration

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Abbreviations

%CV	co-efficient of variability
AB	Alamar Blue
b.w	body weight
BBB	blood brain barrier
C1-4	C-toxins
CNS	central nervous system
d.p.c	day(s) post coitum
dcSTX	decarbamoylated
DMEM	Dulbecco;s Modified Eagle Medium
EB #	EB day
EBs	embryoid body
EFSA	European Food Safety Authority
EROD	ethoxyresorufin-*0*-deethylase
FBS	foetal bovine serum
GPx	glutathione peroxidase
GTXs	gonyuatoxins
HAB	harmful algal blooms
hERG	human ether-a-go-go
IF	intermediate filament
LDH	lactate dehydrogenase
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
MAP2	microtubule-associated protein 2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

$NA_v1.1$-1.9	α-subunits
neoSTX	neosaxitoxin
P-loop	pore loop
PAC	powdered activated carbon
PLL	poly-L-lysine
PNS	peripheral nervous system
PROD	penthoxyresorufin-*O*-deethylase
PSP	paralytic shell fish poisoning
PSTs	paralytic shellfish toxins
RA	retinoic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
S1-S6	transmembrane segments
SOD	superoxide dismutase
ß1-4	ß-subunits
STX	saxitoxin
STXeq	saxitoxin equivalents
TTX	tetrodotoxin
VGCC	voltage-gated calcium channel
VGSC	voltage-gated sodium channel

1. Low Dose Extended Exposure to Saxitoxin and its potential neurodevelopmental effects: a review.

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1.1 Abstract

Saxitoxin (STX) and its analogs, the paralytic shellfish toxins (PSTs), are a group of potent neurotoxins well known for their role in acute paralytic poisoning by preventing the generation of action potentials in neuronal cells. They are found in both marine and freshwater environments globally and although acute exposure from the former has previously received more attention, low dose extended exposure from both sources is possible and to date has not been investigated. Given the known role of cellular electrical activity in neurodevelopment this pattern of exposure may be a significant public health concern. Additionally, the presence of PSTs is likely to be an ongoing and possibly increasing problem in the future. This review examines the neurodevelopmental toxicity of STX, the risk of extended or repeated exposure to doses with neurodevelopmental effects, the potential implications of this exposure and briefly, the steps taken and difficulties faced in preventing exposure.

1.2 Introduction

STX is a neurotoxin most commonly known for its role in paralytic shellfish poisoning (PSP) and the majority of past research has been focused on acute exposure from this source. However, there is also the potential of extended exposure to low doses of the toxin, from this source and others, and this pattern of exposure has not been thoroughly investigated. While exposure to high doses of STX can be fatal, low dose extended exposure has the potential to affect neurodevelopment through the action of the toxin at voltage-gated sodium channels (VGSCs) which have been shown to play an important role in a developing nervous system.

Low dose extended exposure from shellfish may occur in communities which rely heavily on a seafood diet, consuming more than the daily average and for considerable periods of time. Additionally at risk are small isolated coastal communities who may harvest untested shellfish. It has been shown that tolerance can occur in some populations [1] so that communities harvesting untested shellfish may be exposed to concentrations higher than safety guidelines, which would cause acute poisoning in a sensitive individual, but would go unnoticed in a tolerant individual. In such cases, while acute poisonings may not occur more subtle low dose adverse effects may be taking place.

The toxin is also produced at lower concentrations by freshwater cyanobacteria which can be found in fresh water sources from which drinking water is sourced [2]. Based on human data from acute paralytic

shellfish poisoning events, a drinking water guideline value of 3μg/L has been established in multiple countries including Australia, Brazil and New Zealand [3, 4] and there have been no acute poisonings to date [5].

There are multiple water treatment methods available for the removal of the cyanobacterial cells responsible for the production of STX and the extracellular dissolved toxin [2]. The percentage of each removed depends on the methods used and while consumers are protected from acute toxicity, low dose exposure can still occur and could occur for extended periods of time considering the duration of algal blooms. Although extended low dose extended exposure is more likely via drinking water there is no guideline for long-term exposure as there has been no research into this pattern of exposure.

It has been suggested that the predicted future climatic changes of global warming such as increased water temperatures, nutrient loading and stratification as well as altered hydrology will favor freshwater cyanobacterial growth and give cyanobacteria a competitive advantage over other phytoplankton. In fact harmful algal blooms in marine settings have already been seen to increase since the 1970s [6, 7] and an increase in total cyanobacteria numbers and individual algal bloom durations has been noted since the 1980s [8]. Additionally the link between algal blooms and eutrophication has been noted since the 1980s [9].

1.3 STX and its analogs

STX itself is part of a large group of analogs collectively known as the paralytic shellfish toxins (PSTs) or in some cases the saxitoxins. This group has a long history with human poisonings dating back to at least 1793[10]. Despite this history the toxin was not isolated until 1957 from the butter clam *Saxidomus giganteus*, after which the toxin is named [11]. Due to its noncrystalline and highly polar nature, the structure of the toxin was not determined for almost another 20 years [12]. STX is one of the most potent natural toxins known, with a place on Schedule 1 of the Chemical Weapons Convention [13].

The PST analogs all share a 3,4,6-trialkyl tetrahydropurine skeleton with two guanidinium groups [12](Figure 1.1). Variations to the side chains give the analogs varying levels of toxicity and the analogs are grouped depending on their side chain variations. STX and neoSTX are non-sulfated, the Gonyautoxins (GTXs) mono-sulfated and the C-toxins di-sulfated, each respectively less toxic than STX. Further variants include decarbamoyls. Authors have described up to 57 analogs [14], with the most common shown in Table 1.1. STX is highly polar and stable in solution [11] while the c-toxins and GTXs are not particularly stable and can degrade to produce more toxic analogs [15]. So while the concentration of individual analogs will vary the group of toxins can persist in water for long periods of time, therefore there is a potential for extended exposure periods.

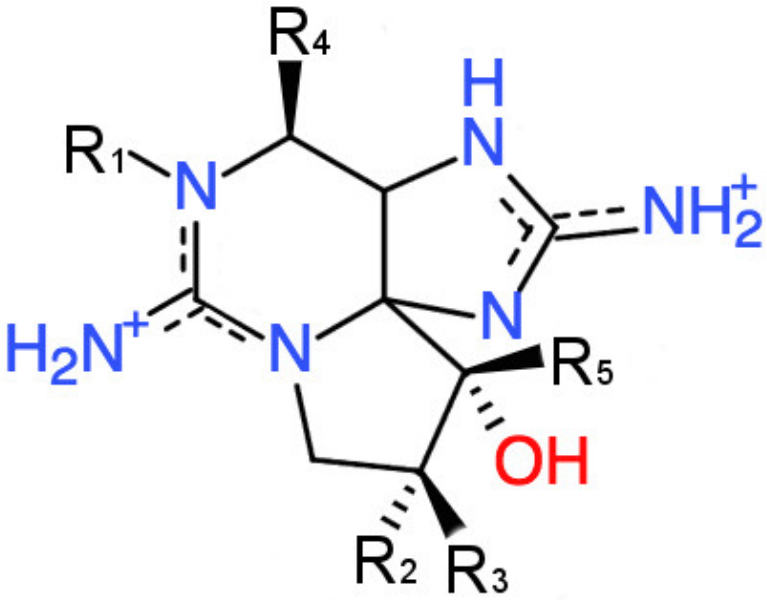

Figure 1.1 The tetrahydropurine skeleton of STX and its analogs

, for R group substituents see Table 1.1.

Toxin	R1	R2	R3	R4	R5	Mouse Bioassay[1]
STX	H	H	H	$OCONH_2$	OH	1656 - 2483
neoSTX	OH	H	H	$OCONH_2$	OH	1038 - 2300
Gonyuatoxins						
GTX1	OH	H	OSO_3^-	$OCONH_2$	OH	1638 - 2468
GTX2	H	H	OSO_3^-	$OCONH_2$	OH	793 - 1028
GTX3	H	OSO_3^-	H	$OCONH_2$	OH	1463 - 2234
GTX4	OH	OSO_3^-	H	$OCONH_2$	OH	1803
GTX5	H	H	H	$OCONHSO_3^-$	OH	160
GTX6	OH	H	H	$OCONHSO_3^-$	OH	Not done
C-toxins						
C1	H	H	OSO_3^-	$OCONHSO_3^-$	OH	15-17
C2	H	OSO_3^-	H	$OCONHSO_3^-$	OH	237 - 329
C3	OH	H	OSO_3^-	$OCONHSO_3^-$	OH	33
C4	OH	OSO_3^-	H	$OCONHSO_3^-$	OH	143
Decarbamoylated						
dcSTX	H	H	H	OH	OH	955 - 1274
dcneoSTX	OH	H	H	OH	OH	Not done
dcGTX1	OH	H	OSO_3^-	OH	OH	Not done
dcGTX2	H	H	OSO_3^-	OH	OH	1617
dcGTX3	H	OSO_3^-	H	OH	OH	1872
dcGTX4	OH	OSO_3^-	H	OH	OH	Not done

[1]Mouse units/μmol where 1 mouse unit is the amount of toxin what kills a 20g mouse in 15minutes

Table 1.1 Side chain variations to the tetrahydropurine skeleton for STX and its analogs.

Adapted from [13, 14].

1.4 Sources of STX Exposure

As mentioned, STX and its analogs are produced in both marine and freshwater environments. It was originally thought that both marine dinoflagellates and freshwater cyanobacteria produce PSTs by the same biosynthetic pathway [16], which is mediated by the *stx* gene cluster in cyanobacteria [17, 18] but the genes responsible for toxin production in dinoflagellates are now thought to be quite different [19]. It has been recently shown that only a small number of the proteins involved in the biosynthetic pathway in cyanobacteria are present in dinoflagellates, so that the later steps in the pathway may be performed by different reactions or enzymes [20].

The reason why either of these organisms produce the toxin is unknown although there are theories, the most common being defense but from what is not known. Another theory suggests a relationship between intracellular Na^+ levels and STX production, where toxic strains of cyanobacteria would be at an advantage under conditions of high pH or Na^+ stress[21, 22]. Based on genetic analysis it has been suggested that the *stx* gene cluster could have emerged at least 2,100 Ma, in an environment significantly different to today. At that time organisms had not evolved VGSCs, the most well known target of the PSTs, and so another theory is that the evolutionary predecessor of the channel, the potassium channels, could have been the target of the toxin [23].

1.4.1 Marine production of STX

The most well-known and researched source of the STXs are the marine dinoflagellates from the genera *Alexandrium, Gymnodinium* and *Pyrodinium* [24-26]. The marine dinoflagellates produce PSTs which are consumed by invertebrates such as shellfish, crustaceans and molluscs, and rarely fish [27]. The majority of these vectors are not affected by the toxin. The toxins become concentrated by these vectors and are ingested by shellfish consumers, causing PSP [14]. There are strict regulatory limits for this source of exposure, a maximum of 80μg STXequivalents/100g of shellfish tissue, and there are prevention programs set up globally as the number of countries affected by harmful algal blooms (HAB) containing PSTs increases [7, 28]. This regulatory limit was first established in the 1930s, originally in Mouse Units, before the structure and toxicology of the toxins were fully understood, but has been effective in protecting seafood consumers since [29]. The vast majority of poisonings in recent times have been a result of harvesting shellfish from quarantined areas, knowingly or unknowingly, or from harvesting untested shellfish which could be a problem for small isolated coastal communities. More recently acute exposure to the PSTs has been reassessed by the European Food Safety Authority (EFSA) [30] and a guideline of 7.5μg STXeq/100g has been suggested, considerably less than the regulatory limit stated above.

1.4.2 Freshwater production of STX

STX and its analogs are also produced by freshwater cyanobacteria including those in the genera *Dolichospermum* (previously *Anabaenea* [31]), *Cylindrospermopsis, Aphanizomenon, Plankotothrix* and *Lyngbya* [32-38]. Their presence in freshwater has been recorded globally with different genera being responsible in each country. There are toxic and non-toxic sub populations of these cyanobacteria and there is additional variation in which toxic analogs each produce (Table 1.2).

With seasonal changes in cell numbers, the toxin concentration can vary in fresh water bodies throughout the year and between water sources (<1-approx. 23μg/l raw water, 5-3400μg/g dry weight)[1, 2]. The toxins can persist in water for several months and analogs can undergo transformation to become more toxic, so that concentrations of individual analogs continuously vary [39]. Even when there is a predominance of less toxic analogs, the toxicity of a mixture of these toxins is still largely defined by the lower concentrations of more toxic analogs [40].

Country	Toxic species	PST analogs produced
Australia	*Dolichospermum*[1]	C1, C2, GTX2, GTX3 and STX
Brazil	*Cylindrospermopsis raciborskii*[2]	neoSTX and STX
USA	*Aphanizomenon flos-aquae*[3] *Lyngbya wollei*[4]	neoSTX and STX dc-GTX2, dc-GTX3, dcSTX and 6 unidentified analogs
Portugal	*Aphanizomenon flos-aquae*[5,6] *Aphanizomenon gracile*[7]	neoSTX, dcSTX, STX, GTX6, GTX5, GTX4, GTX1, GTX3 neoSTX and STX
Germany	*Aphanizomenon gracile*[8]	GTX5, STX, dcSTX and neoSTX
Italy	*Planktothrix* sp.[9]	STX
China	*Aphanizomenon flos-aquae*[10,11]	STX, neoSTX, GTX5, dcSTX and dcGTX3

Table 1.2 Locations and characteristics of freshwater PST production

. 1[33] 2[34] 3[35] 4[32] 5[37] 6[41] 7[42] 8[43] 9[38] 10[44] 11[45].

PSP producing cyanobacteria have been shown to carry the *stx* gene cluster. It is suggested that the *stx* gene cluster has a single origin amongst cyanobacteria because only a small number of genes have recombinations between species [18], and that this strong conservation of the stx gene cluster over time shows it has a vital role in the survival of the species that possess it [23].

As mentioned above the predicted future climatic changes of global warming will favor the growth of cyanobacteria. These favored growth conditions could have implications for the frequency, timing, size, duration and distribution of algal blooms, with the general consensus that these will all increase [41-44]. While the effect this will have on toxin production is still unclear it is predicted that toxin production will increase as growth conditions are more favorable [45] which will subsequently alter the concentrations of toxin reaching drinking water and the patterns of exposure to toxin, having potentially serious consequences for drinking water [46].

1.5 STX activity

The most well-known action of STX is its ability to block VGSCs, but it has also been described to act at calcium [47, 48] and human ether-a-go-go (hERG) potassium channels [49]. Furthermore it binds to the protein saxiphilin, a transferrin-like protein originally isolated from the North

American bullfrog, *Rana castesbeiana* [50] as well as a pufferfish protein isolated from *Fugu pardalis* [51].

1.5.1 Activity at sodium channels

The VGSCs are transmembrane proteins made up of an α-subunit and one or more β-subunits. The α-subunit is made up of 4 identical domains each with 6 transmembrane segments [52](Figure 1.2). The domains are arranged around the central pore of the channel with segment 6 forming the inner surface. STX binds to Site 1, which is located on the hairpin-like pore loop (P-loop) between segments 5 and 6 [53], blocking the pore of the channel and preventing the flow of Na ions [14, 54] with the gating of VGSC having no effect on the blocking action of STX. It has been suggested that there are two predominantly negatively charged rings of carboxylated amino acids within the proximity of the channel pore on the P-loop and this is where the positively charged guanidinium groups of STX bind, with the IC_{50} shown to be 1.2±0.2nM in wild type rat sodium channel type 2 [55]. Alterations such as the addition of a hydroxyl group or a sulfate group can change the affinity of the toxin for the channel giving the different analogs their varying toxicity [13].

Nine different isoforms of the α-subunit have been described ($Na_v1.1$ to $Na_v1.9$) each with varying distribution throughout the human body and each with varying sensitivity to STX [56-58].

Isoforms $Na_v1.1$, $Na_v1.2$, $Na_v1.3$ and $Na_v1.6$ are found mainly in the central nervous system, while $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ are found in the peripheral nervous system. These groups of isoforms are known as 'brain type' or 'neuronal-type' sodium channels. $Na_v1.4$ and $Na_v1.5$ are both non-neuronal with $Na_v1.4$ mainly found in skeletal muscle and $Na_v1.5$ being cardiac-exclusive [54]. Hence different regions of the body will have varying sensitivity to the PSTs. Isoform expression additionally varies during development so that sensitivity to the PSTs may vary over time [59](Table 1.3).

While all of the α-subunit isoforms have been classified as either sensitive, resistant or insensitive to tetrodotoxin (TTX), a structurally different toxin which also acts on VGSC, this has not been done for all isoforms in regards to the PSTs. Testing the sensitivity of each isoform would be beneficial to better understanding which regions of the body would be more at risk to extended low dose exposure to STX and during which stages of life.

Isoform	Spatial expression[1]	Temporal expression
$Na_v1.1$	CNS	Postnatal to adult nervous tissue[1]
$Na_v1.2$	CNS	Embryonic to adult nervous tissue[1,2]
$Na_v1.3$	CNS	Embryonic to adult nervous tissue[1]
Nav1.4	Skeletal muscle	Postnatal to adult cardiac tissue[3]
Nav1.5	Cardiac muscle	Embryonic to Adult cardiac tissue[1,3]
Nav1.6	CNS	Adult nervous system[2]
Nav1.7	PNS	Adult nervous system[1]
Nav1.8	PNS	Embryonic to adult nerve cells[4]
Nav1.9	PNS	Embryonic to adult nerve cells[4]

Table 1.3 Spatial and temporal expression of the VGSC isoforms.

CNS = central nervous system, PNS = peripheral nervous system 1[64] 2[65] 3[61] 4[66]

The β-subunits, which can be found in excitable and non-excitable cells in both the central and peripheral nervous system, are made up of an extracellular domain, a transmembrane domain and an intracellular domain [54, 60]. Each α-subunit is can be associated with one covalently linked β-subunit (β2 or β4) and one non-covalently linked β-subunit (β1 or β3). The β-subunits have a role as regulatory proteins which can modulate the expression of VGSC on the cell surface, and can modulate cellular migration as well as neurite extension [60]. As mentioned STX binds to the α-subunit and is not known to have any direct effect on the β-subunit.

The primary function of VGSC is to produce action potentials. Neurons and other excitable cells have an electrical potential between the intra- and extracellular environments caused by an imbalance of ions across the cellular membrane. By eliminating and reestablishing this potential, neurons can transmit electrical signals along their axons. Removal of the potential due to certain stimuli opens the VGSC allowing an inflow of Na^+ ions rapidly depolarizing the cell, closure of the channel then allows for repolarization by the oubaine sensitive Na/K antiporter and a return to steady state completing the action potential. By blocking the movement of Na ions through the channel, STX halts the generation of action potentials and the neurons lose their ability to transmit electrical impulses.

1.5.2 Activity at calcium channels

Voltage-gated calcium channels are also transmembrane proteins and can be made up of four components, α_1-subunit, β-subunit, $\alpha_2\delta$-subunit and calmodulin [61]. The α_1-subunit forms the channel pore similarly to the VGSC, having 4 homologous domains each with 6 transmembrane segments and having a similar amino-acid sequence [62](Figure 1.2). Again there are multiple isoforms of this channel with varying distribution and sensitivity to STX. The high-voltage activated channels including the L-, P/Q- and R-type are made up of all four components mentioned above whereas the T-type which is low-voltage activated needs only the α_1-subunit to function [61]. N- and P-type calcium channels are mainly neuronal, with T- and L-type channels being muscular and neuronal [13].

It has been shown that STX blocks calcium currents in bullfrog sympathetic neurons expressing N-type channels with an IC_{50} of 400nm [47] and more recently it was shown to partially block L-type calcium channels with an IC_{50} of 0.3μM [48]. It has been suggested that STX's binding site on this channel is external and may be similar to the VGSC binding site [48]. This suggested binding site is not surprising considering the similarities in the α_1 -subunit of the calcium channel and the α_1 -subunit of the VGSC. This same group demonstrated that greater concentrations of STX were required for the same level of channel blockade in calcium channels as in neuronal VGSCs, but lower than those required for cardiac VSGCs.

The channel is responsible for control of cellular calcium entry and has a role in multiple cellular functions including, but not limited to, muscle contraction, neurotransmitter and hormone release and calcium-dependent gene regulation [61, 62].

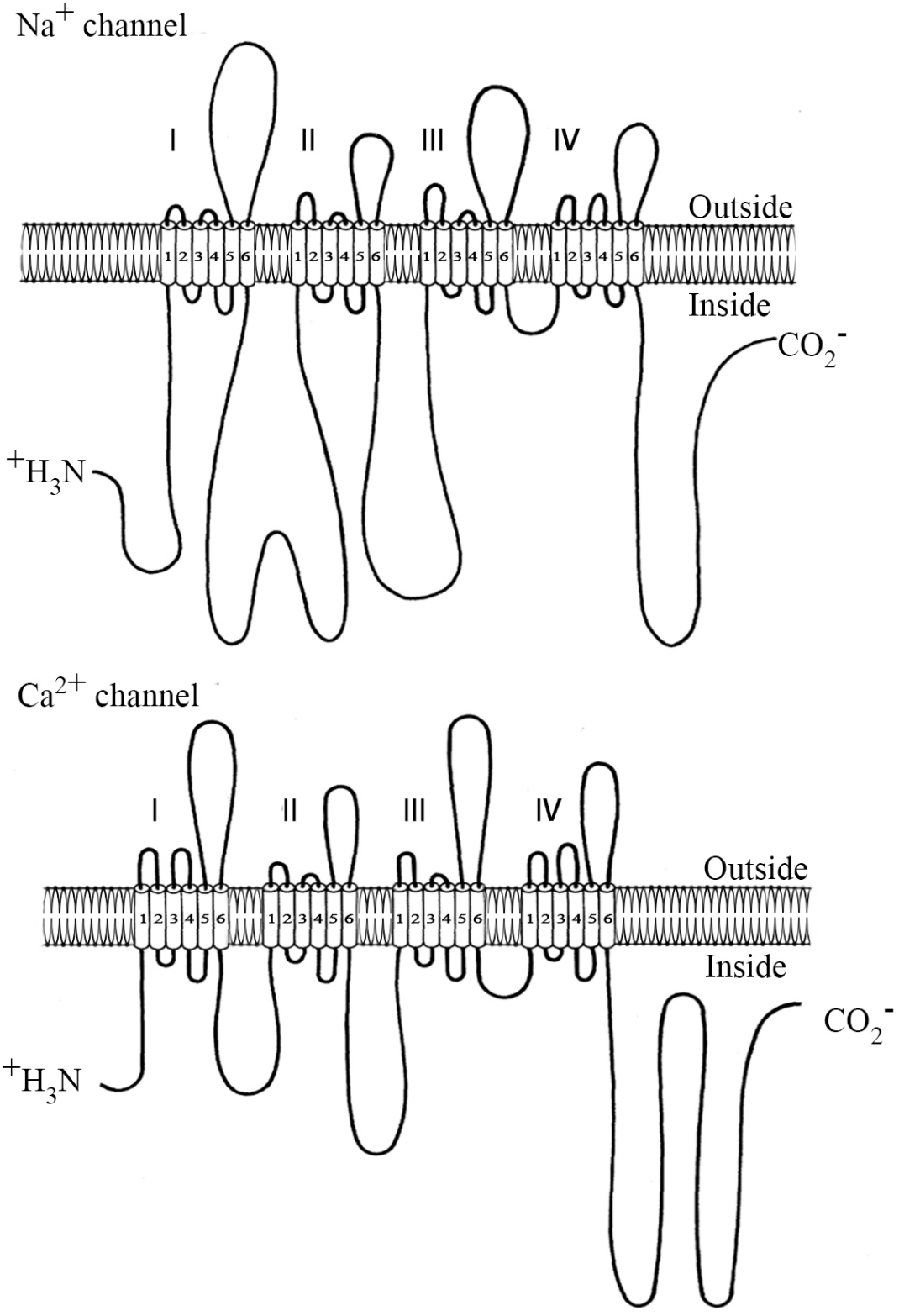

Figure 1.2 Arrangement of the sodium and calcium channel α-subunit ,

showing the 4 identical domains each with 6 transmembrane segments, redrawn from [70].

1.5.3 Activity at potassium channels

Also a transmembrane channel, the hERG K^+ channel is made up of four α-subunits each with six transmembrane segments (S1-S6) [63]. S1-S4 of each α-subunit make up the voltage sensing domain surrounding S5-S6 which make up the central pore domain [49]. The hERG K^+ channel has small structural differences from the majority of other voltage gated K^+ channels which could explain why PSPs have only been seen to bind hERG K+ channels [64].

Mainly found in cardiac myocytes and neurons, the hERG K^+ channel controls the duration of cardiac action potentials through repolarization and has been linked to syndromes which can cause fatal cardiac arrhythmias [65-67]. The channel has also been detected in human pancreatic β-cells [68]. Unlike in VGSCs, STX did not act by blocking the ion pore of this channel, but instead modified the gating of it in HEK-293 cells. Stronger depolarization was required to open channels bound with STX and upon repolarization the channels closed much faster [49]. Modification of the channel was membrane potential dependent, at -50mV EC50 was 5.6μM and at 50mV EC50 was 0.3μM.

1.6 Exposure to STX

1.6.1 Acute exposure to STX

Acute exposure to STX occurs as a result of eating contaminated shellfish, potentially resulting in PSP, or from ingestion of contaminated

freshwater. As mentioned above no acute human poisonings have been recorded from freshwater, yet there have been livestock deaths [69, 70]. However, close to 2,000 PSP cases are reported yearly around the world with a fatality rate of 15% [6] and fatalities have been recorded in marine life also [71].

Following exposure to STX, action potentials in nerve and muscle fibers are interrupted. Symptoms can be both gastrointestinal and neurological including; paraesthesia, nausea, vomiting, incoordination, diarrhea, weakness, ataxia, shortness of breath, dysarthria, dysphagia, hypotension and, depending on the amount of toxin consumed, can result in complete paralysis of the victim and death via respiratory depression [28, 72]. The outcome of PSP is variable between individuals, for example children have been shown to be more susceptible with a higher mortality rate [72-74].

Death can occur within an hour of a lethal dose and there is currently no antidote, with the only sufficient treatment being artificial respiration [13]. PSP victims may also be treated with activated charcoal to remove any unabsorbed toxin [75]. Victims surviving more than 24 hours have much greater chances of making a full recovery [76].

It has also been shown that PSTs can be bioaccumulated in the freshwater environment in the same fashion as the marine source, but with different vectors. The filter-feeding freshwater mussel *Alathyria condola* Iredale has been shown to accumulate PSTs when fed *A.*

circinalis for 7 days and extracts from mussels produced typical PSP symptoms in the mouse bioassay [77]. Whilst these mussels are not commonly consumed, they are occasionally consumed by populations of Indigenous Australians and they are consumed by aquatic animals.

Exposure to PSTs can also occur during recreational water use. In addition to the PSTs cyanobacteria are also known to produce lipopolysaccharide (LPS) endotoxins [78] so that during recreational exposure adverse reactions may be a result of the PSTs and/or LPS. In a large international cohort study the majority of symptoms recorded following recreational exposure to cyanobacteria were mild respiratory, however there was not a significant increase compared to controls [79].

1.6.2 Pharmacokinetics of STX

The effects of exposure to the PSTs obviously depends on the pharmacokinetics of the toxins. Beginning with absorption, GTX 2/3 epimers have been shown to be readily transported across the epithelium by both paracellular and transcellular routes [80-82] in Caco-2 and IEC-6 cells as well as in human intestinal samples. Analysis of tissue and body fluids taken post-mortem from victims of PSP show distribution of the toxin in the brain, bile, cerebrospinal fluid, liver, spleen, heart, thyroid and adrenal glands, kidneys, pancreas and lungs [83]. Pharmacokinetic studies in cats again showed distribution throughout the body in the brain, medulla oblongata, liver and spleen, with the largest percentage of the

dose reaching the spleen [84]. This widespread distribution explains both the neurological and gastrointestinal symptoms resulting from PSP and demonstrates the capability of the PSTs to cross the blood-brain barrier. It is worth noting that these studies used high concentrations of STX which caused death in the cats and the post-mortem analysis were from victims of PSP, therefore receiving high concentrations of toxin also. This questions whether the ability of STX to cross the blood-brain barrier would be seen at low concentrations. Cardiovascular burdens and hypercapnia can significantly alter the permeability of the blood-brain barrier, conditions which have been recorded following exposure to high doses of STX [85, 86], but if these effects are not seen at low doses of STX exposure will the toxin still be able to cross the blood-brain barrier? If not, the toxin will still exert its effect on the peripheral nervous system.

In a more recent study in cats using GTX2/3 epimers it was shown that the toxins are mainly excreted via glomerular filtration [84] which supports the earlier work in cats and rats which suggested excretion through urine [81, 87]. There is some difference in opinion as to whether or not the PSTs are metabolized. In the same pharmacokinetic study mentioned above only GTX2 and GTX3 were recovered in urine and following incubation of GTX2/3 epimers with cat liver supernatant 100% of the toxin was recovered, suggesting that no metabolism took place [80]. Similarly the urine of rats treated with dihydrosaxitoxin did not show any other toxin analogs [87].

In contrast the post-mortem analysis mentioned above recorded STX and GTX1-5 in the gastric content, yet in the body fluids, spleen and pancreas neoSTX was recorded, as was dcSTX in liver, kidneys and lungs suggesting that transformation of the analogs does occur in humans [83]. The same group has shown that GTX2 and GTX3 can undergo oxidation and subsequent glucuronidation by human liver microsomes in vitro, converting them to four major metabolites; glucuronic-GTX3, glucuronic-GTX2, GTX4 and GTX1 [88] and have also shown the same conversion of neoSTX and STX to glucuronic-neoSTX and glucuronic-STX respectively [89]. In each study they recovered only 6% and 15% of the original PSTs respectively, suggesting that the majority are converted. This glucoronidation metabolic pathway could explain the increased sensitivity to PSTs in children as glucoronidation develops during childhood [90] and the earlier work which did not record any metabolism of the PSTs in cats is likely to be due to the fact that glucouronidation is not a metabolic pathway found in cats [14].

Metabolism is also supported by findings from another post-mortem analysis of a PSP victim where the toxin profile of the gut varied greatly from that of the urine [91]. The gut content was made up primarily of STX with lesser amounts of GTX2, GTX3 and neoSTX, whereas in the urine only half of the toxin found was STX; GTX2 and GTX3 were almost completely removed and there was an increase in neoSTX and dcSTX. The group proposed that the N1 group of STX underwent oxidation for the conversion to neoSTX and hydrolysis of the carbamoyl group for

conversion to dcSTX. The different toxin profile seen post-mortem compared to the microsome results could suggest that further metabolism occurs in the kidney or bladder.

As mentioned above the toxicity of a mix of toxins is largely defined by the more toxic analogs even if they are at a lower concentration in comparison to the less toxic analogs [40]. Therefore, it is worth noting that the effect of metabolism on the overall toxicity of a mix of PSPs analogs may be quite small.

1.6.3 Chronic exposure to STX

To date there has been little research into the reproductive, teratogenic, genotoxic or carcinogenic effects of the PSTs [5, 92] despite extended low dose exposure being a possibility.

The majority of work that has been done on extended exposure to STXs was carried out in amphibians and fish. It was shown that extended exposure to STX in zebrafish had significant adverse effects on morphology, growth and survival [93]. Similar morphological effects were seen by another group using zebrafish who also observed an increase in mortality during larval development and altered hatching time, this altered hatching time was also seen in axolotl [94]. Due to the high concentrations used in both experiments (10-500μg/L STX), it is likely that the effects on growth and survival were a result of paralysis and reduced feeding, which was noted in the treated fish of the first study. Concentrations used in both

experiments were much greater than those which people are likely to be exposed to for an extended period so that no suggestions can be made of what would be expected to result in humans, but it does highlight the lack of research into this pattern of exposure.

When exposed to lower concentration of PSTs both fish and mammalian models have shown significant changes to antioxidant mechanisms[95] as well as DNA damage suggestive of apoptosis [96]. Antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) were significantly reduced in the liver of mice exposed to sub-lethal concentrations of GTX2/3 (300μg//kg and 200μg/kg respectively) as were the xenobiotic metabolizing enzymes ethoxyresorufin-*0*-deethylase (EROD) and penthoxyresorufin-*0*-deethylase (PROD).The less toxic analogs C1/2 (450μg/kg and 1960μg/kg respectively) also significantly lowered the activity of PROD and GPx [95].

Further, rats ingesting STX spiked drinking water (3μg/L and 9μg/L in drinking) for 30 days had significant changes to antioxidant mechanisms in both the brain and liver including total antioxidant capacity and levels of reactive oxygen species, lipid hydroperoxides and oxidative enzymes [97]. At the drinking water guideline of 3μg/L there was a reduction in the total antioxidant capacity in the hippocampus but not in the pre-frontal cortex suggesting that not all areas of the brain would be affected equally following extended low-dose exposure to the PSTs.

These changes in antioxidant mechanisms could cause an increase in oxidative stress and have adverse effects on neurodevelopment as the brain has an increased vulnerability to oxidative stress and fewer defenses [98], especially the developing brain having increased metabolic demand required for growth and fewer glia [99], which have been shown to protect neurons from oxidative stress [100]. How the PSTs mediate these changes to antioxidant mechanisms is unknown.

By reducing the action of metabolizing enzymes STX may alter the metabolism of other compounds or it may very well reduce the rate of its own metabolism creating the potential for enzyme polymorphisms to result in inter-individual differences in response to STX. This supports the idea that the PSTs may have adverse effects not just in the nervous system.

Non neurological adverse effects have been seen in wild fish following extended exposure to PSTs (4.6 - 83.44 STX eq ng/L) [101]. These included epithelial hyperplasia in the gills as well as necrosis and Melano-macrophage centers in the liver.

Another potential outcome of extended exposure is alterations in neurogenesis as electrical activity has been described as playing an important role in normal CNS development [102]. Therefore the activity of low concentrations of STX at voltage gated ion channels could have implications for neurodevelopment. For example, disruption of electrical activity using TTX has been shown to inhibit neurite outgrowth mediated by the β1 subunit of the VGSC channel [102], triggering neuronal

apoptosis mediated by a rise in postsynaptic response to glutamate and reduced ability to clear intracellular calcium [103]. In *Drosophila*, an increase in sprouting from the transverse nerve onto muscle fibers at neuromuscular junctions has been shown to occur following treatment with TTX [104].Furthermore it has also been shown that STX administration can change levels of neuroactive amino acids in multiple brain regions of rats, yet the particular channel-blocking action of STX causing these changes is unknown [105].

Calcium channels have also been implicated in proper neuronal development. These channels have been shown to have an important part in the activity-dependent refinement of connections at neuromuscular junctions in *Drosophila* [106], suggesting that STX's action at these ion channels could have a further effect on development.

1.7 Measures taken and difficulties faced in preventing exposure

As there is no antidote and limited treatment available for PSP, it is of great importance to prevent exposure. As mentioned there is a guideline of 80μg STXeq/100g of shellfish and this has been very successful at preventing acute lethal poisoning from this source, however low dose non-symptomatic exposure can still occur. Given that the assumed daily consumption of shellfish is 200g this would equate to a total of 160μg STX eq or 2.7μg STXeq/kg body weight (b.w) for a 60kg adult. In comparison drinking water guidelines are 3μg/L STX and the assumed daily

consumption of water is 2L which would equate to a total of 6μg STXeq or 0.1μg STXeq/kg b.w for a 60kg adult making it even more precautionary in preventing exposure. Additionally, as the 2L is an assumed daily consumption and STX does not accumulate in the body the actual concentration of STX an individual is exposed to at any one time is likely to be less than this.

As mentioned above the EFSA has suggested lowering the regulatory guideline for shellfish to 7.5μg STXeq/100g of shellfish, based on an estimated no-observed-adverse-effect level of 0.5μg STXeq/kg b.w. They have also noted the large variation in the amount of shellfish consumed in different populations and that the current guideline for shellfish exposure is not protective enough and should be adjusted to protect people consuming large amounts of shellfish [30]. These communities which consume large amounts of shellfish could be at risk of extended low dose exposure to STX if consumed on a daily basis.

The first approach to preventing PST exposure in drinking water is prevention of algal blooms that produce the toxins. Reservoir mixing or destratification can be used to reduce cyanobacterial growths. Secondly freshwater can successfully be treated so that PST concentrations are below guidelines of 3μg/L. The best approach is to have a multi barrier method so that both intracellular and extracellular toxins can be removed. As STX is predominantly intracellular, treatments such as coagulation and filtration are effective in removing cells and therefore the majority of toxins,

then the addition of powdered activated carbon (PAC) is used to remove extracellular toxins, and chlorination for disinfection. The combination of coagulation, PAC and ultrafiltration has been shown to remove all cyanobacterial cells and 90% of intracellular PSTs, but extracellular PSTs could not be successfully quantified due to their low concentration [107]. Other treatments can include granular activated carbon filtration and ozonation [107, 108].

Although treatments are successful at lowering the concentration of STX below guidelines, low concentrations may still be present in drinking water. For example spot checks following treatment recorded PSPs close to 0.5μg/L [2]. This same group has shown that PSTs can persist in water for consecutive months.

To guarantee the safety of drinking water, methods used to quantify concentrations of PSTs must be accurate and precise, for which there are numerous techniques each with their own advantages and drawbacks [109]. With further research into this pattern of exposure it is also important to have methods available which can accurately and precisely quantitate concentrations of PSTs, if the investigation of low-dose extended exposure to the PSPs resulted in lowering of the guidelines, current analytical methods may not be sensitive enough.

1.8 Conclusion

The PSTs are a group of potent neurotoxins with a long history of poisoning from marine sources with their production by freshwater cyanobacteria being a more recent discovery. The public health risk of acute exposure via marine sources is well managed with regulatory guidelines and monitoring programs and acute poisoning from freshwater is unlikely due successful water treatment. So while acute PST poisoning is uncommon low dose extended exposure, about which very little is known, is still a public health risk

This pattern of exposure is of particular concern considering the action of STX on multiple ion channels and the role of ion channels and their associated electrical activity in neurodevelopment. It is likely that the PSTs will not just be causing an inhibition of action potentials via their activity at VGSC, but could in fact be preventing more complex cellular pathways orchestrated by these multiple ion channels. Evidence of this has already been seen with significant effects on cellular antioxidant mechanisms.

Additionally, it is likely that production of PSTs will be an ongoing concern as cyanobacteria growth has been predicted to increase with the predicted future changes to climate. This together with the lack of research and possible neurodevelopmental effects of extended exposure makes low dose STX in freshwater a significant public health concern which must be addressed.

1.9 References

1. Chorus, I.B., Jamie, *Toxic Cyanobacteria in Water: A guide to their public consequences, monitoring and management*. 1999, London: E & FN Spon.

2. Hoeger, S.J., et al., *Occurrence and elimination of cyanobacterial toxins in two Australian drinking water treatment plants.* Toxicon, 2004. **43**(6): p. 639-649.

3. Burch, M., *Effective doses, guidelines & regulations*, in *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs*, H.K. Hudnell, Editor. 2008, Springer New York. p. 831-853.

4. ADWG, *Australian Drinking Water Guidelines Paper 6 National Water Quality Management Strategy.* 2011(National Health and Medical Research Council, National Resource Management Ministerial Council, Commonwealth of Australia, Canberra).

5. Zegura, B., Straser, A., and Filipic, M., *Genotoxicity and potential carcinogenicity of cyanobacterial toxins - a review.* Mutat Res, 2011. **727**(1-2): p. 16-41.

6. Hallegraeff, G.M., *A review of harmful algal blooms and their apparent global increase*.* Phycologia, 1993. **32**(2): p. 79-99.

7. Van Dolah, F.M., *Marine algal toxins: origins, health effects, and their increased occurrence.* Environ Health Perspect, 2000. **108 Suppl 1**: p. 133-41.

8. Croome R, W.L., Henderson B, Oliver R, Vilizzi L., Paul W and McInerney P, *River Murray Water Quality Monitoring Program: Phytoplankton Data Trend Analysis 1980-2008. Final Report prepared for the Murray-Darling Basin Authority by The Murray-Darling Freshwater Research Centre.* MDFRC Publication, 2011. **June**.

9. Anderson, D.M., Glibert, P.M., and Burkholder, J.M., *Harmful Algal Blooms and Eutrophication: Nutrient Sources, Composition, and Consequences.* Estuaries, 2002. **25**(4): p. 704-726.

10. Price, D.W., Kizer, K.W., and Hansgen, K.H., *California's paralytic shellfish poisoning prevention program 1927-89.* Journal of Shellfish Research, 1991. **10**(1): p. 119-146.

11. Schantz, E.J., et al., *Paralytic Shellfish Poison. VI. A Procedure for the Isolation and Purification of the Poison from Toxic Clam and Mussel Tissues.* Journal of the American Chemical Society, 1957. **79**(19): p. 5230-5235.

12. Schantz, E.J., et al., *Letter: The structure of saxitoxin.* J Am Chem Soc, 1975. **97**(5): p. 1238.

13. Llewellyn, L.E., *Saxitoxin, a toxic marine natural product that targets a multitude of receptors.* Nat Prod Rep, 2006. **23**(2): p. 200-22.

14. Wiese, M., et al., *Neurotoxic alkaloids: saxitoxin and its analogs.* Mar Drugs, 2010. **8**(7): p. 2185-211.

15. Fanger, G.R., Jones, J.R., and Maue, R.A., *Differential regulation of neuronal sodium channel expression by endogenous and exogenous tyrosine kinase receptors expressed in rat pheochromocytoma cells.* J Neurosci, 1995. **15**(1 Pt 1): p. 202-13.

16. Shimizu, Y., *Microalgal metabolites.* Chemical Reviews, 1993. **93**(5): p. 1685-1698.

17. Kellmann, R., et al., *Biosynthetic intermediate analysis and functional homology reveal a saxitoxin gene cluster in cyanobacteria.* Appl Environ Microbiol, 2008. **74**(13): p. 4044-53.

18. Mihali, T.K., Kellmann, R., and Neilan, B.A., *Characterisation of the paralytic shellfish toxin biosynthesis gene clusters in Anabaena circinalis AWQC131C and Aphanizomenon sp. NH-5.* BMC Biochem, 2009. **10**: p.8.

19. Yang, I., et al., *Comparative gene expression in toxic versus non-toxic strains of the marine dinoflagellate Alexandrium minutum.* BMC Genomics, 2010. **11**: p. 248.

20. Hackett, J.D., et al., *Evolution of Saxitoxin Synthesis in Cyanobacteria and Dinoflagellates.* Molecular Biology and Evolution, 2013. **30**(1): p. 70-78.

21. Pomati, F., Burns, B.P., and Neilan, B.A., *Identification of an Na(+)-dependent transporter associated with saxitoxin-producing strains of the cyanobacterium Anabaena circinalis.* Appl Environ Microbiol, 2004. **70**(8): p. 4711-9.

22. Pomati, F., et al., *Interactions between intracellular Na+ levels and saxitoxin production in Cylindrospermopsis raciborskii T3.* Microbiology, 2004. **150**(2): p. 455-461.

23. Murray, S.A., Mihali, T.K., and Neilan, B.A., *Extraordinary Conservation, Gene Loss, and Positive Selection in the Evolution of an Ancient Neurotoxin.* Molecular Biology and Evolution, 2011. **28**(3): p. 1173-1182.

24. Harada, T., Oshima, Y., and Yasumoto, T., *Structures of Two Paralytic Shellfish Toxins, Gonyautoxins V and VI, Isolated from a Tropical Dinoflagellate, Pyrodinium bahamense var. compressa.* Agricultural and Biological Chemistry, 1982. **46**(7): p. 1861-1864.

25. Lefebvre, K.A., et al., *Characterization of intracellular and extracellular saxitoxin levels in both field and cultured Alexandrium spp. samples from Sequim Bay, Washington.* Mar Drugs, 2008. **6**(2): p. 103-16.

26. Oshima, Y., et al., *Dinoflagellate Gymnodinium catenatum as the source of paralytic shellfish toxins in Tasmanian shellfish.* Toxicon, 1987. **25**(10): p. 1105-1111.

27. Deeds, J.R., et al., *Non-traditional vectors for paralytic shellfish poisoning.* Mar Drugs, 2008. **6**(2): p. 308-48.

28. Etheridge, S.M., *Paralytic shellfish poisoning: Seafood safety and human health perspectives.* Toxicon, 2010. **56**(2): p. 108-122.

29. Wekell, J.C., Hurst, J., and Lefebvre, K.A., *The origin of the regulatory limits for PSP and ASP toxins in shellfish.* Journal of Shellfish Research, 2004. **23**(3): p. 927-930.

30. EFSA, *Marine biotoxins in shellfish - Saxitoxin group. Scientific Opinion of the Panel on Contaminants in the Food Chain.* The EFSA Journal, 2009. **1019**: p. 1-76.

31. Wacklin, P., Hoffman, L., and Komárek, J., *Nomenclatural validation of the genetically revised cyanobacterial genusDolichospermum(RALFS ex BORNET et FLAHAULT) comb. nova.* Fottea, 2009. **9**(1): p. 59-64.

32. Carmichael, W.W., et al., *Evidence for paralytic shellfish poisons in the freshwater cyanobacterium Lyngbya wollei (Farlow ex Gomont) comb. nov.* Applied and Environmental Microbiology, 1997. **63**(8): p. 3104-10.

33. Humpage, A.R., et al., *Paralytic shellfish poisons from Australian cyanobacterial blooms.* Aust. J. Mar. Freshwater Res., 1994. **45**(5): p. 761-771.

34. Lagos, N., et al., *The first evidence of paralytic shellfish toxins in the freshwater cyanobacterium Cylindrospermopsis raciborskii, isolated from Brazil.* Toxicon, 1999. **37**(10): p. 1359-1373.

35. Mahmood, N.A. and Carmichael, W.W., *Paralytic shellfish poisons produced by the freshwater cyanobacterium Aphanizomenon flos-aquae NH-5.* Toxicon, 1986. **24**(2): p. 175-186.

36. Onodera, H., et al., *New saxitoxin analogues from the freshwater filamentous cyanobacterium Lyngbya wollei.* Natural Toxins, 1997. **5**(4): p. 146-151.

37. Pereira, P., et al., *Paralytic shellfish toxins in the freshwater cyanobacterium Aphanizomenon flos-aquae, isolated from Montargil reservoir, Portugal.* Toxicon, 2000. **38**(12): p. 1689-1702.

38. Pomati, F., et al., *The Freshwater Cyanobacterium Planktothrix Sp. FP1: Molecular Identification and Detection of Paralytic Shellfish Poisoning Toxins.* Journal of Phycology, 2000. **36**(3): p. 553-562.

39. Jones, G.J. and Negri, A.P., *Persistence and degradation of cyanobacterial paralytic shellfish poisons (PSPs) in freshwaters.* Water Research, 1997. **31**(3): p. 525-533.

40. Llewellyn, L.E., *The Behavior of Mixtures of Paralytic Shellfish Toxins in Competitive Binding Assays.* Chemical Research in Toxicology, 2006. **19**(5): p. 661-667.

41. Markensten, H., Moore, K., and Persson, I., *Simulated lake phytoplankton composition shifts toward cyanobacteria dominance in a future warmer climate.* Ecol Appl, 2010. **20**(3): p. 752-67.

42. Carey, C.C., et al., *Eco-physiological adaptations that favour freshwater cyanobacteria in a changing climate.* Water Res, 2012. **46**(5): p. 1394-407.

43. Jones, I.D., et al., *Increases in lake phytoplankton biomass caused by future climate-driven changes to seasonal river flow.* Global Change Biology, 2011. **17**(5): p. 1809-1820.

44. Elliott, J.A., *Is the future blue-green? A review of the current model predictions of how climate change could affect pelagic freshwater cyanobacteria.* Water Res, 2012. **46**(5): p. 1364-71.

45. Reichwaldt, E.S. and Ghadouani, A., *Effects of rainfall patterns on toxic cyanobacterial blooms in a changing climate: between simplistic scenarios and complex dynamics.* Water Res, 2012. **46**(5): p. 1372-93.

46. Paerl, H.W. and Paul, V.J., *Climate change: links to global expansion of harmful cyanobacteria.* Water Res, 2012. **46**(5): p. 1349-63.

47. Jones, S.W. and Marks, T.N., *Calcium currents in bullfrog sympathetic neurons. I. Activation kinetics and pharmacology.* J Gen Physiol, 1989. **94**(1): p. 151-67.

48. Su, Z., et al., *Saxitoxin blocks L-type ICa.* J Pharmacol Exp Ther, 2004. **308**(1): p. 324-9.

49. Wang, J., Salata, J.J., and Bennett, P.B., *Saxitoxin is a gating modifier of HERG K+ channels.* J Gen Physiol, 2003. **121**(6): p. 583-98.

50. Llewellyn, L.E., Bell, P.M., and Moczydlowski, E.G., *Phylogenetic Survey of Soluble Saxitoxin-Binding Activity in Pursuit of the Function and*

Molecular Evolution of Saxiphilin, a Relative of Transferrin. Proceedings: Biological Sciences, 1997. **264**(1383): p. 891-902.

51. Yotsu-Yamashita, M., et al., *Purification, characterization, and cDNA cloning of a novel soluble saxitoxin and tetrodotoxin binding protein from plasma of the puffer fish, Fugu pardalis.* European Journal of Biochemistry, 2001. **268**(22): p. 5937-5946.

52. Sato, C., et al., *The voltage-sensitive sodium channel is a bell-shaped molecule with several cavities.* Nature, 2001. **409**(6823): p. 1047-1051.

53. Stevens, M., Peigneur, S., and Tytgat, J., *Neurotoxins and their binding areas on voltage-gated sodium channels.* Front Pharmacol, 2011. **2**: p. 71.

54. Savio-Galimberti, E., Gollob, M.H., and Darbar, D., *Voltage-gated sodium channels: biophysics, pharmacology, and related channelopathies.* Front Pharmacol, 2012. **3**: p. 124.

55. Terlau, H., et al., *Mapping the site of block by tetrodotoxin and saxitoxin of sodium channel II.* FEBS Letters, 1991. **293**(1,2): p. 93-96.

56. Shinohara, R., et al., *Synthesis of Skeletal Analogues of Saxitoxin Derivatives and Evaluation of Their Inhibitory Activity on Sodium Ion Channels NaV1.4 and NaV1.5.* Chemistry – A European Journal, 2011. **17**(43): p. 12144-12152.

57. Gilchrist, J. and Bosmans, F., *Animal toxins can alter the function of Nav1.8 and Nav1.9.* Toxins (Basel), 2012. **4**(8): p. 620-32.

58. Walker, J.R., et al., *Marked difference in saxitoxin and tetrodotoxin affinity for the human nociceptive voltage-gated sodium channel (Nav1.7).* Proceedings of the National Academy of Sciences, 2012. **109**(44): p. 18102-18107.

59. Shafer, T.J., Meyer, D.A., and Crofton, K.M., *Developmental Neurotoxicity of Pyrethroid Insecticides: Critical Review and Future Research Needs.* Environmental Health Perspectives, 2005. **113**(2): p. 123-136.

60. Patino, G.A. and Isom, L.L., *Electrophysiology and beyond: multiple roles of Na+ channel beta subunits in development and disease.* Neurosci Lett, 2010. **486**(2): p. 53-9.

61. Findeisen, F. and Minor Jr, D.L., *Progress in the structural understanding of voltage-gated calcium channel (CaV) function and modulation.* Channels, 2010. **4**(6).

62. Tanabe, T., et al., *Primary structure of the receptor for calcium channel blockers from skeletal muscle.* Nature, 1987. **328**(6128): p. 313-318.

63. S. Hari Narayana Moorthy, N., J. Ramos, M., and A. Fernandes, P., *Human Ether-a-Go-Go-Related Gene Channel Blockers and its Structural Analysis for Drug Design.* Current Drug Targets, 2013. **14**(1): p. 102-113.

64. Du, L., et al., *A novel structure-based virtual screening model for the hERG channel blockers.* Biochemical and Biophysical Research Communications, 2007. **355**(4): p. 889-894.

65. Curran, M.E., et al., *A molecular basis for cardiac arrhythmia: HERG mutations cause long QT syndrome.* Cell, 1995. **80**(5): p. 795-803.

66. Sanguinetti, M.C., et al., *A mechanistic link between an inherited and an acquird cardiac arrthytmia: HERG encodes the IKr potassium channel.* Cell, 1995. **81**(2): p. 299-307.

67. Schönherr, R., et al., *Functional role of the slow activation property of ERG K+ channels.* European Journal of Neuroscience, 1999. **11**(3): p. 753-760.

68. Rosati, B., et al., *Glucose- and arginine-induced insulin secretion by human pancreatic Œ≤-cells: the role of HERG K+ channels in firing and release.* The FASEB Journal, 2000. **14**(15): p. 2601-2610.

69. McBarron, E.J., et al., *Toxicity To Livestock of the Blue-Green Alga Anabaena Circinalis.* Australian Veterinary Journal, 1975. **51**(12): p. 587-588.

70. Negri, A.P., Jones, G.J., and Hindmarsh, M., *Sheep mortality associated with paralytic shellfish poisons from the cyanobacterium Anabaena circinalis.* Toxicon, 1995. **33**(10): p. 1321-1329.

71. Reyero, M., et al., *Evidence of saxitoxin derivatives as causative agents in the 1997 mass mortality of monk seals in the Cape Blanc Peninsula.* Natural Toxins, 1999. **7**(6): p. 311-315.

72. de Carvalho, M., et al., *Paralytic shellfish poisoning: clinical and electrophysiological observations.* J Neurol, 1998. **245**(8): p. 551-4.

73. Rodrigue, D.C., et al., *Lethal paralytic shellfish poisoning in Guatemala.* Am J Trop Med Hyg, 1990. **42**(3): p. 267-71.

74. Gessner, B.D., Middaugh, J.P., and Doucette, G.J., *Paralytic shellfish poisoning in Kodiak, Alaska.* West J Med, 1997. **167**(5): p. 351-3.

75. Pearson, L., et al., *On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin.* Marine Drugs, 2010. **8**(5): p. 1650-1680.

76. Long, R.R., Sargent, J.C., and Hammer, K., *Paralytic shellfish poisoning: A case report and serial electrophysiologic observations.* Neurology, 1990. **40**(8): p. 1310-1312.

77. Negri, A.P. and Jones, G.J., *Bioaccumulation of paralytic shellfish poisoning (PSP) toxins from the cyanobacterium Anabaena circinalis by the freshwater mussel Alathyria condola.* Toxicon, 1995. **33**(5): p. 667-78.

78. Papageorgiou, J., et al., *Extraction of cyanobacterial endotoxin.* Environmental Toxicology, 2004. **19**(1): p. 82-87.

79. Stewart, I., et al., *Epidemiology of recreational exposure to freshwater cyanobacteria--an international prospective cohort study.* BMC Public Health, 2006. **6**: p. 93.

80. Andrinolo, D., et al., *Transport of the organic cations gonyautoxin 2/3 epimers, a paralytic shellfish poison toxin, through the human and rat intestinal epitheliums.* Toxicon, 2002. **40**(10): p. 1389-1397.

81. Andrinolo, D., et al., *Toxicokinetics and toxicodynamics of gonyautoxins after an oral toxin dose in cats.* Toxicon, 2002. **40**(6): p. 699-709.

82. Torres, R., et al., *GTX 2/3 Epimers Permeate the Intestine through a Paracellular Pathway.* The Journal of Toxicological Sciences, 2007. **32**(3): p. 241-248.

83. Garcia, C., et al., *Paralytic shellfish poisoning: post-mortem analysis of tissue and body fluid samples from human victims in the Patagonia fjords.* Toxicon, 2004. **43**(2): p. 149-158.

84. Andrinolo, D., Michea, L.F., and Lagos, N., *Toxic effects, pharmacokinetics and clearance of saxitoxin, a component of paralytic shellfish poison (PSP), in cats.* Toxicon, 1999. **37**(3): p. 447-464.

85. Evans, C.A., et al., *The effect of hypercapnia on a blood-brain barrier mechanism in foetal and new-born sheep.* J Physiol, 1976. **255**(3): p. 701-14.

86. Chang, F.-C.T., et al., *Central and peripheral cardio-respiratory effects of saxitoxin (STX) in urethane-anesthetized guinea-pigs.* Toxicon, 1993. **31**(5): p. 645-664.

87. Hines, H.B., Naseem, S.M., and Wannemacher Jr, R.W., *[3H]-Saxitoxinol metabolism and elimination in the rat.* Toxicon, 1993. **31**(7): p. 905-908.

88. García, C., et al., *Evidence of in vitro glucuronidation and enzymatic transformation of paralytic shellfish toxins by healthy human liver microsomes fraction.* Toxicon, 2009. **53**(2): p. 206-213.

89. Garcia, C., et al., *Route of metabolization and detoxication of paralytic shellfish toxins in humans.* Toxicon, 2010. **55**(1): p. 135-44.

90. Strassburg, C.P., et al., *Developmental aspects of human hepatic drug glucuronidation in young children and adults.* Gut, 2002. **50**(2): p. 259-265.

91. Llewellyn, L.E., et al., *Post-mortem analysis of samples from a human victim of a fatal poisoning caused by the xanthid crab, Zosimus aeneus.* Toxicon, 2002. **40**(10): p. 1463-1469.

92. Munday, R. and Reeve, J., *Risk assessment of shellfish toxins.* Toxins (Basel), 2013. **5**(11): p. 2109-37.

93. Lefebvre, K.A., Trainer, V.L., and Scholz, N.L., *Morphological abnormalities and sensorimotor deficits in larval fish exposed to dissolved saxitoxin.* Aquatic Toxicology, 2004. **66**(2): p. 159-170.

94. Oberemm, A., et al., *Effects of cyanobacterial toxins and aqueous crude extracts of cyanobacteria on the development of fish and amphibians.* Environmental Toxicology, 1999. **14**(1): p. 77-88.

95. Hong, H.-z., Lam, P.K.S., and Hsieh, D.P.H., *Interactions of paralytic shellfish toxins with xenobiotic-metabolizing and antioxidant enzymes in rodents.* Toxicon, 2003. **42**(4): p. 425-431.

96. da Silva, C.A., et al., *First report about saxitoxins in freshwater fish Hoplias malabaricus through trophic exposure.* Toxicon, 2011. **57**(1): p. 141-147.

97. Ramos, P.B., et al., *Oxidative stress in rats induced by consumption of saxitoxin contaminated drink water.* Harmful Algae, 2014. **37**(0): p. 68-74.

98. Gupta, R.C., *Brain Regional Heterogeneity and Toxicological Mechanisms of Organophosphates and Carbamates.* Toxicology Mechanisms & Methods, 2004. **14**(3): p. 103-143.

99. Song, C., Kanthasamy, A., and Kanthasamy, A., *Chapter 63 - Cell signaling mechanisms in developmental neurotoxicity*, in *Reproductive and Developmental Toxicology*, R.C. Gupta, Editor. 2011, Academic Press: San Diego. p. 835-845.

100. Tanaka, J., et al., *Astrocytes prevent neuronal death induced by reactive oxygen and nitrogen species.* Glia, 1999. **28**(2): p. 85-96.

101. Clemente, Z., et al., *Analyses of paralytic shellfish toxins and biomarkers in a southern Brazilian reservoir.* Toxicon, 2010. **55**(2–3): p. 396-406.

102. Brackenbury, W.J., et al., *Functional reciprocity between Na+ channel Nav1.6 and beta1 subunits in the coordinated regulation of excitability and neurite outgrowth.* Proc Natl Acad Sci U S A, 2010. **107**(5): p. 2283-8.

103. Fishbein, I. and Segal, M., *Miniature synaptic currents become neurotoxic to chronically silenced neurons.* Cereb Cortex, 2007. **17**(6): p. 1292-306.

104. Jarecki, J. and Keshishian, H., *Role of neural activity during synaptogenesis in Drosophila.* The Journal of Neuroscience, 1995. **15**(12): p. 8177-8190.

105. Cianca, R.C.C., et al., *Differential changes of neuroactive amino acids in samples obtained from discrete rat brain regions after systemic administration of saxitoxin.* Neurochemistry International, 2008. **54**(5-6): p. 308-313.

106. Carrillo, R.A., et al., *Presynaptic activity and CaMKII modulate retrograde semaphorin signaling and synaptic refinement.* Neuron, 2010. **68**(1): p. 32-44.

107. Dixon, M.B., et al., *A coagulation-powdered activated carbon-ultrafiltration - Multiple barrier approach for removing toxins from two Australian cyanobacterial blooms.* Journal of Hazardous Materials, 2011. **186**(2,Äì3): p. 1553-1559.

108. Orr, P.T., Jones, G.J., and Hamilton, G.R., *Removal of saxitoxins from drinking water by granular activated carbon, ozone and hydrogen peroxide-implications for compliance with the Australian drinking water guidelines.* Water Res, 2004. **38**(20): p. 4455-61.

109. Humpage, A.R., Magalhaes, V.F., and Froscio, S.M., *Comparison of analytical tools and biological assays for detection of paralytic shellfish poisoning toxins.* Anal Bioanal Chem, 2010. **397**(5): p. 1655-71.

2. Extended Low-Dose Exposure to Saxitoxin Inhibits Neurite Outgrowth in Model Neuronal Cells

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Overall percentage (%)	
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate to include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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2.1 Abstract

The potent neurotoxin saxitoxin (STX) belongs to a group of structurally related analogues produced by both marine and freshwater phytoplankton. The toxins act by blocking voltage-gated sodium channels stopping the inflow of sodium ions and the generation of action potentials. Exposure from marine sources occurs as a result of consuming shellfish which have concentrated the toxins and freshwater exposure can occur from drinking water although there have been no acute poisonings from the latter source to date. Previously the majority of research into this group of toxins, collectively known as the paralytic shellfish toxins, has focused on acute exposure resulting in paralytic shellfish poisoning. While acute exposure guidelines exist for both sources there are no chronic exposure guidelines and there has been minimal research into this pattern of exposure despite the known role of electrical activity in neurogenesis. We aimed to investigate this pattern of exposure and its potential effects on neurodevelopment using model neuronal cells. PC12 and SH-SY5Y cells were exposed to STX (0.25-3μg/l) for seven days, after which time they were stained with TRITC-Phalloidin, to observe adverse morphological effects. Cells exposed to STX had a significant decrease (18 - 85%) in long axon-like projections, instead exhibiting a significant increase in shorter projections classified as filopodia ($p<0.05$). The results suggest that extended low dose exposure to STX can inhibit proper neurite outgrowth at concentrations well below guideline levels for both sources of exposure making it a potential public health concern.

2.2 Introduction

The paralytic shellfish toxins (PSTs) are a group of structurally related neurotoxins produced by both marine and fresh water phytoplankton. The PSTs share a 3,4,6-trialkyl tetrahydropurine skeleton with side chain variations giving up to 57 analogues [1]. These analogues can be grouped depending on their side chain variations including STX, the gonyautoxins (GTX) and the C-toxins. PSTs act by reversibly binding to axonal voltage gated sodium channels (VGSC), blocking the inflow of sodium ions and subsequently the generation of action potentials so that neurons lose their ability to transmit electrical impulses [2]. The result can be numbness, paralysis and even death by respiratory depression. Additionally the PSTs have also been shown to act on calcium and potassium channels [3]. Acute exposure through consumption of marine shellfish has been well researched, giving the toxins their name, yet little is known of the effects of extended low dose exposure from either source.

The most well-known and researched source of the PSTs are the marine dinoflagellates [4], which are consumed by invertebrates such as shellfish, crustaceans and molluscs without being affected by the toxin. The toxins become concentrated by the invertebrates and are then ingested by consumers, causing paralytic shellfish poisoning [1]. There are strict safety guidelines for commercially produced seafood that stipulate a maximum of 80 μg STXeq/100g of shellfish tissue.

Although seafood monitoring programs have been set up globally to prevent acute poisonings, low dose extended exposure may occur in coastal communities who rely heavily on a seafood diet. These communities are likely consuming more than expected daily averages of shellfish and for considerable periods of time. So while acute poisoning may not occur, more subtle low dose adverse effects may be taking place. In fact recently it has been suggested that the current guideline is not protective enough, especially for populations of people consuming large amounts of shellfish, and should be lowered to 7.5μg STXeq/100g of shellfish [5].

In the freshwater environment the PSTs are produced by cyanobacteria [6], a concern for public health as these freshwaters can be the source of drinking water for humans. The cyanobacteria responsible for the production of PSTs as well as the analogues produced vary globally [7]. Drinking water quality guidelines based on acute paralytic shellfish poisoning events suggest a health alert level of 3μg STX eq/l [8] and, while there have been live-stock deaths from drinking untreated water [9], water treatment practices aimed at meeting these guidelines have ensured that there have been no human fatalities from this source. However, although cyanobacterial cell numbers vary seasonally [10] the PSTs can persist in water for several months so that extended low dose exposure below guideline levels is possible [11].

Additionally it has been suggested that the predicted future climatic changes associated with global warming such as increased water temperatures, nutrient loading and stratification, as well as altered rainfall, will favour freshwater cyanobacterial growth. This could have implications for the

frequency, timing, size, duration and distribution of algal blooms [12]. While the effect this will have on toxin production is unknown, it has been predicted that toxin levels will increase as growth conditions become more favourable [13, 14]. Therefore the potential presence of low concentrations of PSTs in drinking water is likely to be a continuing issue in the future.

Given the action of the PSTs at VGSC, one potential but more cryptic outcome of extended exposure is alterations in neurogenesis as electrical activity has been described as playing an important role in normal CNS development [15]. This hypothesis is supported by the fact that the structurally different but similarly acting toxin tetrodotoxin has been shown to disrupt normal CNS development [16].

Therefore we aimed to determine if extended low dose exposure to STX could affect normal neuronal development of axon-like processes by measuring dose-related morphological changes in model neuronal cells. We found that extended low dose exposure to STX inhibited neurite outgrowth in two model neuronal cell lines, PC12 Ordway and SHSY5Y, which have previously been used to study neuronal development [17]. Both cell lines are known to produce neuronal morphology representative of neuronal differentiation, including long axonal-like outgrowths. Additionally PC12 cells are a model of peripheral neurons of the autonomic nervous system while SH-SY5Y cells are a model for neurons from the central nervous system, allowing us to examine the effects on both nervous systems. To our knowledge this is the first paper to look directly at extended low dose exposure to STX and its effects on mammalian neurodevelopment.

2.3 Material and Methods

2.3.1 Materials

All materials were sourced from Sigma Aldrich Australia unless stated otherwise.

2.3.2 Cell culture

PC12 Ordway cells (Donated by Prof. John Piletz; Loyola Medical School, Chicago, USA) and SH-SY5Y cells (American Type Culture Collection, ATCC) were grown in Roswell Park Memorial Institute (RPMI) medium and Dulbecco's Modified Eagle Medium (DMEM), respectively. Each medium was supplemented with 5% fetal calf serum and 2mM L-glutamine, 1% non-essential amino acids, 1000U/ml penicillin and streptomycin. Cells were cultured at 37^{o}C with 5% CO_2 and passaged every 3-4 days. No exogenous growth factors were added.

2.3.3 STX exposure

1x10^5 cells were plated on poly-L-lysine (PLL) coated glass coverslips in their respective growth mediums and incubated overnight (n=3). Cells were treated with STX dihydrochloride (NRC, Canada) (0.25 - 3μg/l) and incubated for 7 days, an exposure time which is considered chronic in these cell lines based on previous studies [18]. Medium and toxin were replaced on day 4.

2.3.4 F-actin staining

Following 7 days of treatment cells were stained with TRITC-Phalloidin, an F-actin stain. Briefly; medium and toxin were removed, cells were fixed in 3.7% formaldehyde for ten minutes and permeabilised in 0.1% Triton-X100 for ten minutes. Non-specific binding sites were blocked with 2% BSA for 1 hour at room temperature and then cells were incubated at room temperature with 1.5μm TRITC-Phalloidin for 15 minutes in the dark. Coverslips were mounted on glass slides with 50% glycerol. Cells were viewed with an Olympus BX50 fluorescence microscope using a narrow green filter (750nm) at 60x objective. Cells were imaged using SPOT image software (Diagnostic Instruments, Inc. Australia).

2.3.5 Morphology analysis

Four fields were randomly chosen per slide, with an average of 36 cells analysed per slide. Using ImageJ (National Institutes of Health, USA) the number of projections per cell and the length of these projections were measured. Cellular projections were classified based on their length. Projections with a length greater than 23μm were classified as axonal like (axons) and projections with a length less than this were classified as filopodia. This length classification was based on a description in which cells were deemed positive for neurite extension if processes were longer than a cell body [9] . F-actin staining distribution was plotted by transecting individual cells.

2.3.6 Statistical analysis

All statistical analyses were performed using GraphPad Prism. Two way ANOVAs with Tukey's multiple comparisons test were used to compare the mean length and classification of projections between treatment groups. One way ANOVA with Tukey's multiple comparisons test was used to compare mean number of projections per cell between treatments groups. Significance $p<0.05$.

2.4 Results

2.4.1 PC12 cell exposure to STX

Over the 7d exposure period control cells developed a neuronal morphology. They had an elongated cell body with long axonal like extensions (Figure 2.1A). There were few projections per cell (2±1 mean ± SEM, Fig. 5) and the vast majority (85%) were classified as axons (Figure 2.6). Following exposure to STX, cells remained in a circular habit with multiple short extensions (Figure 2.1B-G). There was a significant increase in the number of projections per cell at 1*μ*g/l and 2*μ*g/l (Figure 2.5) and the length of these projections was greatly reduced at 0.5 *μ*g/L and above (Figure 2.7). Even the lowest dose of 0.25 *μ*g/l significantly reduced the percentage of projections classified as axons (Figure 2.6). The number of projections per cell increased in a concentration dependent manner up to 2*μ*g/l followed by a decrease at 3*μ*g/l. Of the projections which were classified as axons, their length was significantly shorter following exposure to 0.5 *μ*g/l STX (Figure 2.7) and above compared to the projections classified as axons on control cells.

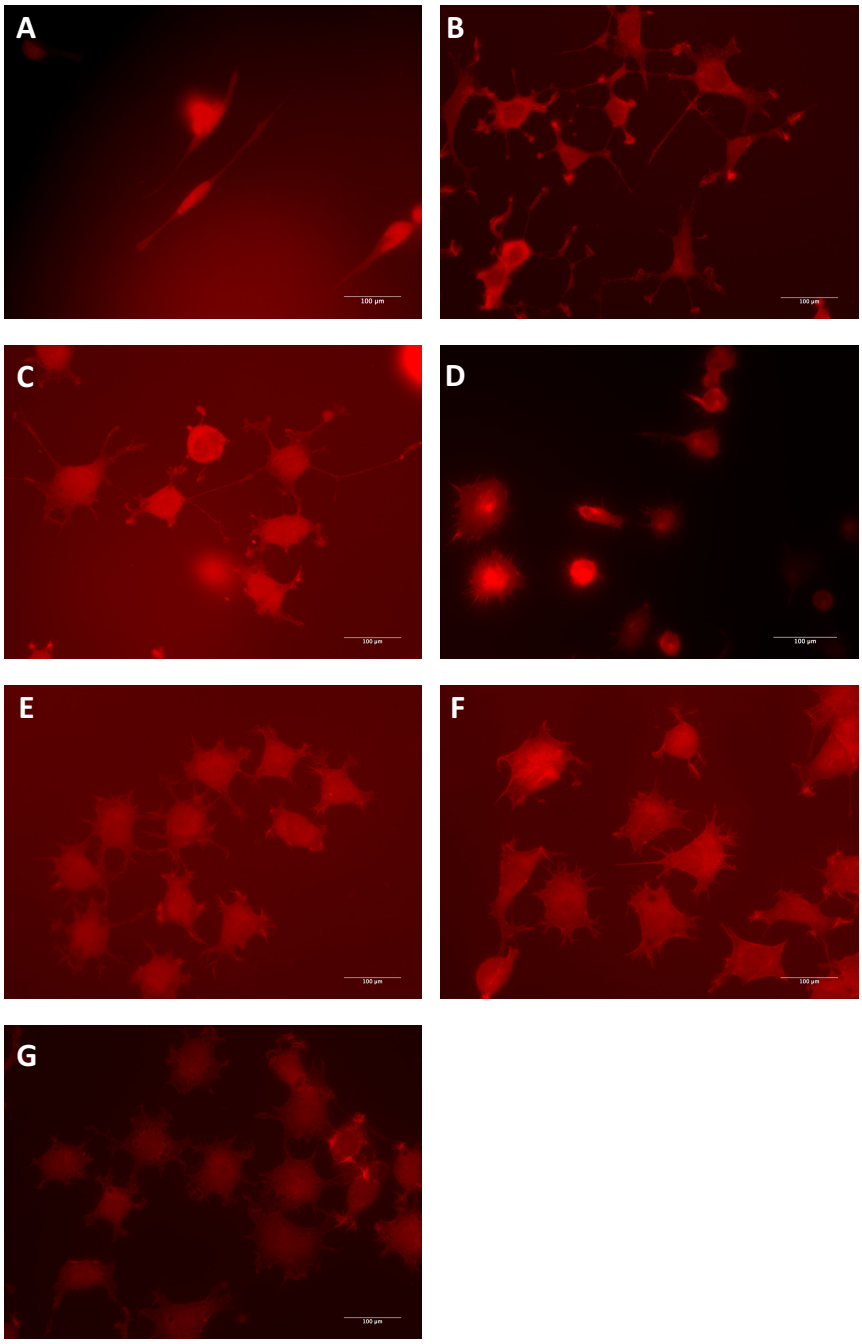

Figure 2.1 Morphology of PC12 cells following exposure to STX.

PC12 cells grown on PLL coated coverslips in 6 well plates were exposed to A. culture medium alone, B. 0.25µg/l, C. 0.5µg/l, D. 0.7µg/l, E. 1µg/l, F. 2µg/l or G. 3µg/l STX with medium and toxin replaced on day 4 before histochemical staining for F-actin on day 7.

Additionally exposure to STX caused changes in patterns of F-actin staining. For control cells F-actin was localised to one pole of the cell body represented by one prominent peak in the cell profile (Figure 2.2A). In comparison exposure to STX lead to disordered F-actin staining with an overall more diffuse distribution within the cells seen as wide peaks in cell profiles (Figure 2.2B-G).

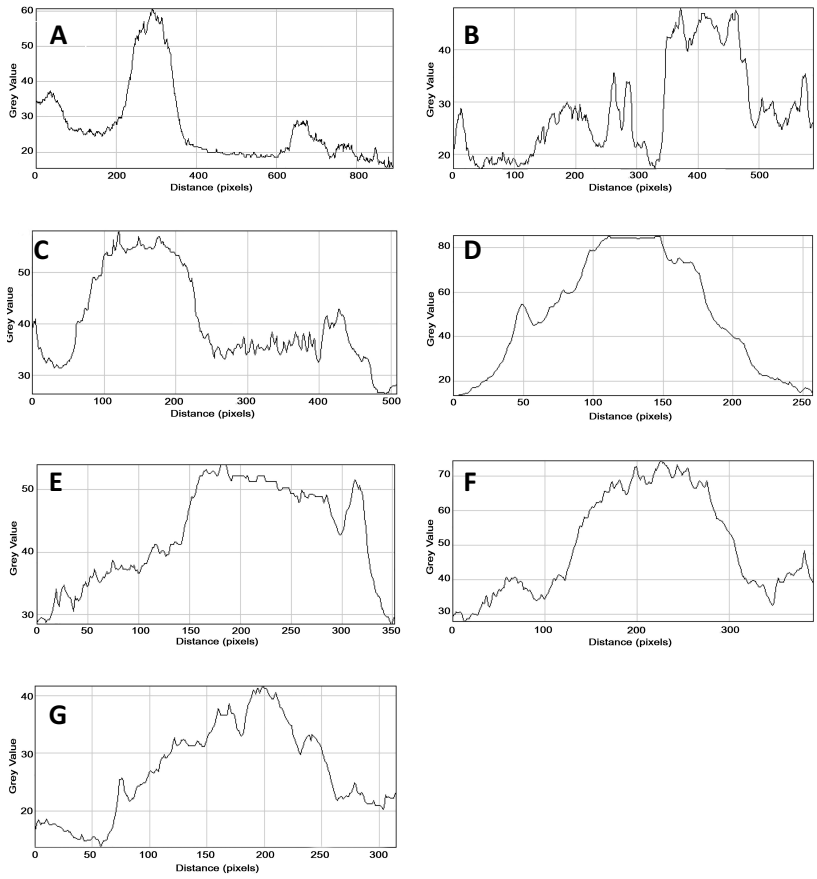

Figure 2.2 F-actin distribution in PC12 cells following exposure to STX.

PC12 cells grown on PLL coated coverslips in 6 well plates were exposed to A. culture medium alone, B. 0.25µg/l, C. 0.5µg/l, D. 0.7µg/l, E. 1µg/l, F. 2µg/l or G. 3µg/l STX with medium and toxin replaced on day 4 before histochemical staining for F-actin on day 7. Distribution of staining was plotted from transects of individual cells.

2.4.2 SHSY5Y cell exposure to STX

Similar to the PC12 cells, control SHSY5Y cells produced a neuronal morphology over the 7d period with long axonal like extensions. In contrast to the PC12 cells, these cells have a pyramidal shaped cell body (Figure 2.3A). Again there were few extensions per control cell (4±1, Figure 2.5) and the majority were classified as axonal like extensions (54±10%, Figure 2.6). Following exposure to STX the same effects were seen as in the PC12 cells. Cells remained in a circular shape with many short extensions (Figure 2.4B-G). Again there was an increase in the number of projections per cell (Figure 2.5), although less than in the PC12 cells with statistically significant effects seen only at 2 μg/l. The percentage of projections classified as axons was also significantly less following exposure to 0.7 μg/l STX and above (Figure 2.6), and those that were classified as axons were shorter (Figure 2.7).

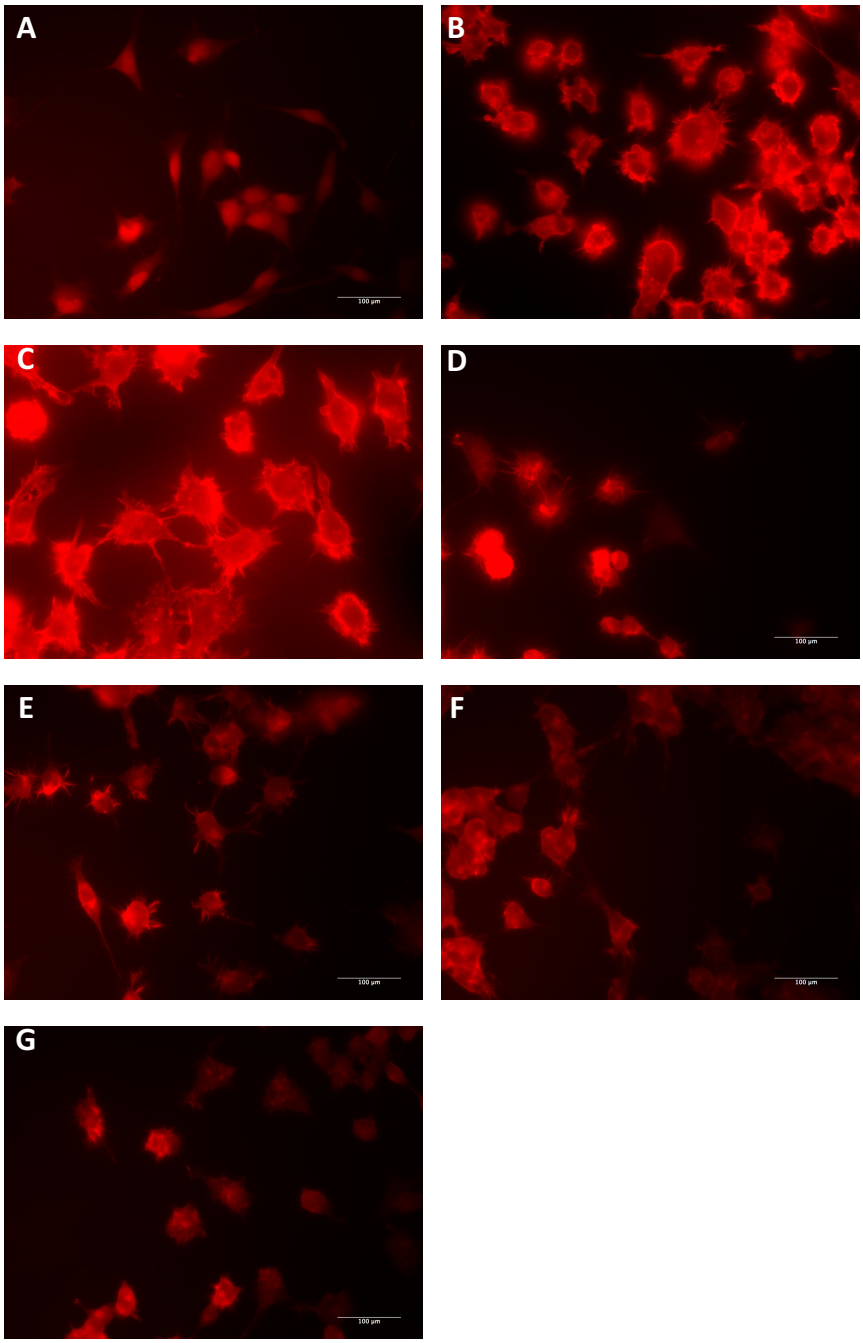

Figure 2.3 Morphology of SHSY5Y cells following exposure to STX.

SHSY5Y cells grown on PLL coated coverslips in 6 well plates were exposed to A. culture medium alone, B. 0.25µg/l, C. 0.5µg/l, D. 0.7µg/l, E. 1µg/l, F. 2µg/l or G. 3µg/l STX with medium and toxin replaced on day 4 before histochemical staining for F-actin on day 7.

As with the PC12 cells there were changes in the pattern of F-actin staining following exposure to STX (Figure 2.4A-G). Again there was a more diffuse distribution of F-actin as well as instances of concentrated staining at the periphery and the base of the projections.

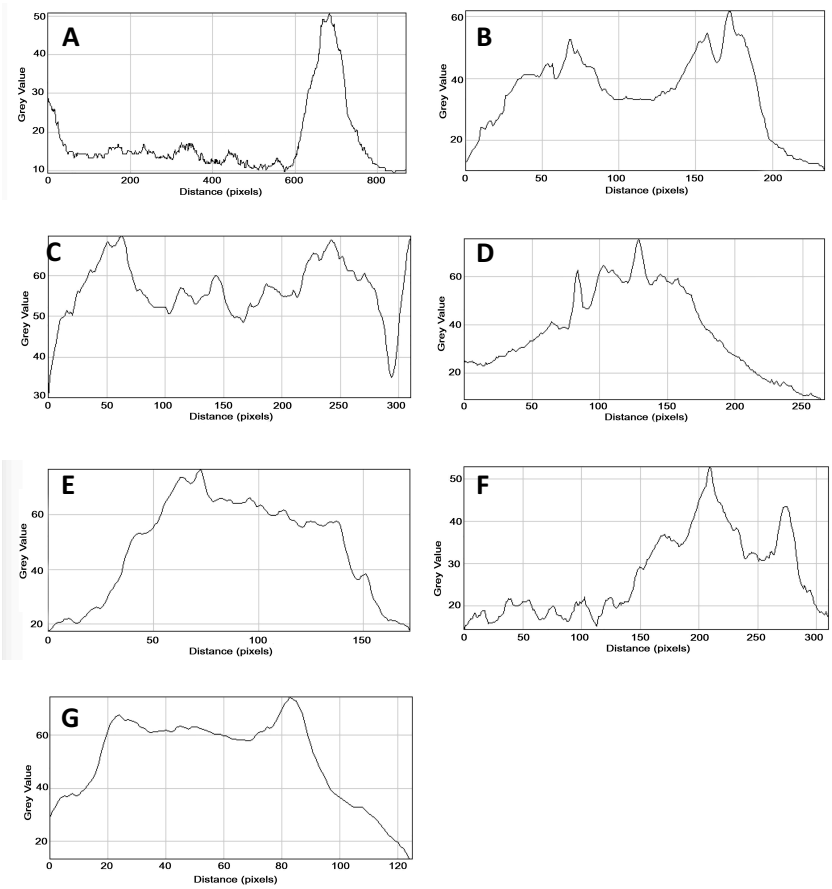

Figure 2.4 F-actin distribution in SHSY5Y cells following exposure to STX.

SHSY5Y cells grown on PLL coated coverslips in 6 well plates were exposed to A. culture medium alone, B. 0.25µg/l, C. 0.5µg/l, D. 0.7µg/l, E. 1µg/l, F. 2µg/l or G. 3µg/l STX with medium and toxin replaced on day 4 before histochemical staining for F-actin on day 7. Distribution of staining was plotted from transects of individual cells.

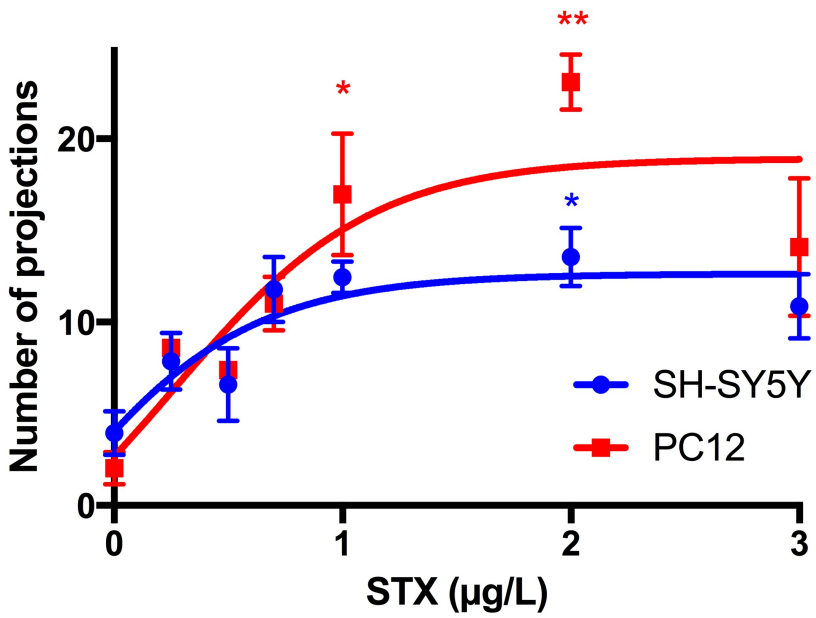

Figure 2.5 Effect of STX on number of projections per cell in PC12 and SH-SY5Y cells.

*Cells grown on PLL coated coverslips in 6 well plates were exposed to culture medium alone or 0.25 - 3µg/l STX with medium and toxin replaced on day 4 before histochemical staining for F-actin on day 7. Data is expressed as mean ± SEM, n=3 separate experiments. Data were subjected to two way ANOVA and Tukey's Multiple comparisons test comparing treatments to vehicle control, *p≤0.05, **p≤0.01*

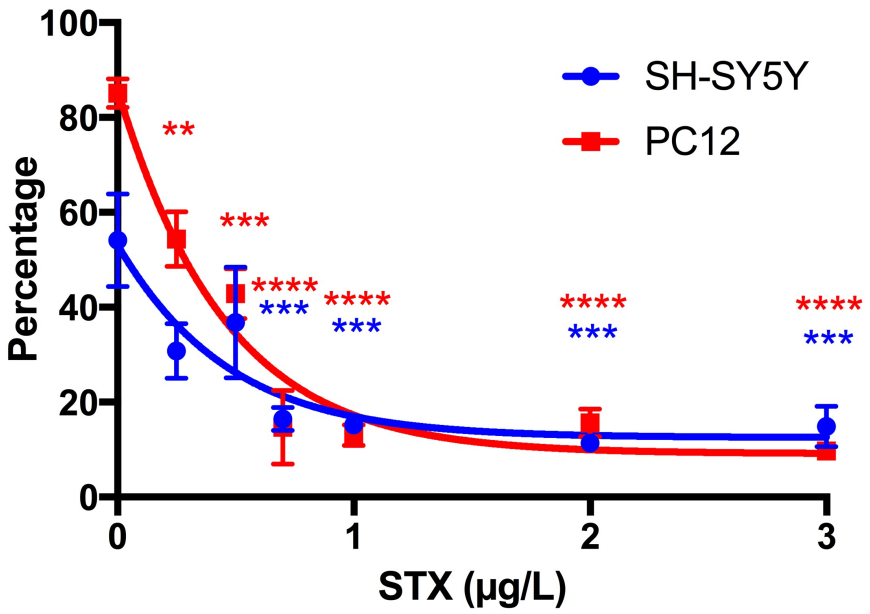

Figure 2.6 Effect of STX on percentage of projections per cell classified as axons in PC12 and SH-SY5Y cells.

*Cells grown on PLL coated coverslips in 6 well plates were exposed to culture medium alone or 0.25 - 3µg/l STX with medium and toxin replaced on day 4 before histochemical staining for F-actin on day 7. Data is expressed as mean ± SEM, n=3 separate experiments. Data were subjected to two way ANOVA and Tukey's Multiple comparisons test comparing treatments to vehicle control, **p≤0.01 ***p≤0.001, ****p≤0.0001. Where no SEM bar is visible, error is smaller than symbol.*

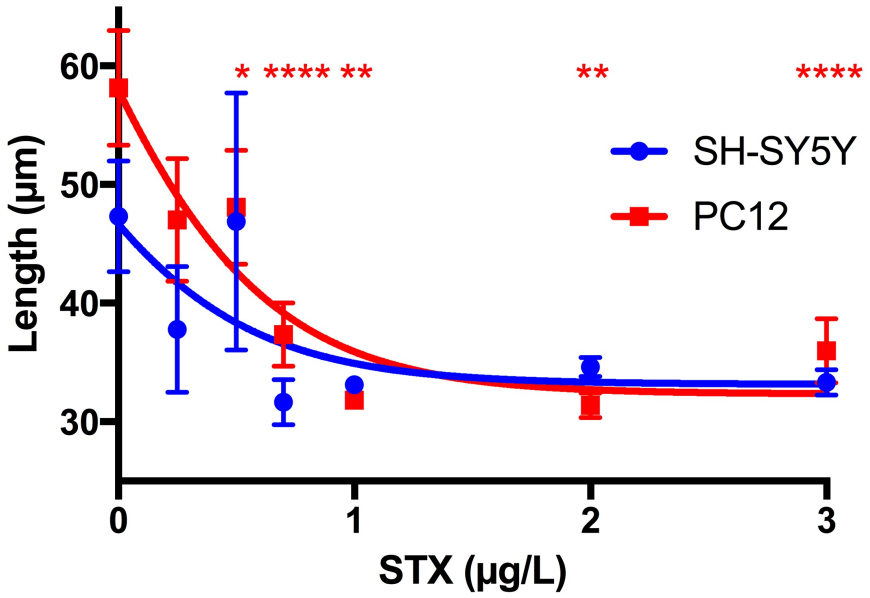

Figure 2.7 Effect of STX on length of projections classified as axons in PC12 and SH-SY5Y cells.

*Cells grown on PLL coated coverslips in 6 well plates were exposed to culture medium alone or. 0.25 - 3µg/l STX with medium and toxin replaced on day 4 before histochemical staining for F-actin on day 7. Data is expressed as mean ± SEM, n=3 separate experiments. Data were subjected to two way ANOVA and Tukey's Multiple comparisons test comparing treatments to vehicle control, *p≤0.05, **p≤0.01, ****p≤0.0001. Where no SEM bar is visible, error is smaller than symbol.*

2.5 Discussion

We aimed to determine if extended low dose exposure to STX would have an effect on normal neuronal development and found that model neuronal cells exposed to STX in this way had inhibited development of axonal like extensions and cells remained in a circular habit. These findings are not entirely surprising as disruption of electrical activity has previously been shown to inhibit proper neuronal development [19] but the fact that dramatic effects were seen at such low concentrations is surprising and raises awareness that this pattern of exposure may be a public health concern.

These findings are significant as the two types of extensions measured, axonal like and filopodia, have different functionality. Axons are capable of neurotransmission whereas filopodia have a role in cellular adhesion, exploration, migration and initiation of neurite outgrowth [20]. This change to a predominance of filopodia after exposure to STX will have functional implications for neuronal cells.

During axonal outgrowth, growth cones made up of filopodia and/or lamellipodia progress through a series of processes which lead to the elongation of axons [21]. The increase in number and decrease in length of filopodia together with the decrease in length of axonal like extensions in this study suggest that the cells exposed to STX are remaining in an immature state or developing at a slower rate.

F-actin is essential for proper axon guidance and is the primary cytoskeleton element which maintains growth [21]. Following exposure to STX there was a disorganisation of the F-actin staining. Given the important role of F-actin in

the initial outgrowth of axons it is likely that STX is indirectly disrupting the proper activity of F-actin and subsequently the number and type of projections seen here.

In comparison to F-actin, microtubules play an important role in axon elongation and are essential to axon structure [21]. It would therefore be beneficial in future studies to stain for both F-actin and tubulin to increase our knowledge of the effect STX is having on axonal outgrowth.

One potential outcome of these adverse effects could be a loss of the cells capability of neurotransmission, or their ability to form axonal links with other neurons and target tissue could be reduced so that neuromuscular development is compromised.

Recently it was shown that following administration of low doses of STX and high doses of tetrodotoxin to cultured primary murine motoneurons (1-10nM and 100nM respectively), axonal outgrowth was reduced [22]. These STX concentrations equate to 0.37-3.7μg/l, taken together with our results this demonstrates the ability of STX to adversely affect multiple cell types including those from the autonomic and central nervous system and motoneurons. Additionally effects were seen in cells from both rodent and humans and appear to represent mechanisms common to both species.

Similarly reduced axonal outgrowth was seen in motoneurons of knockout mice lacking the $Na_v1.9$ VGSC isoform of the sodium channel [22]. Interestingly, the knockout mice with reduced axonal growth showed defects in pain perception, but otherwise developed normally. This raises questions as to how the adverse effects we have seen here in individual cells would

translate to an entire nervous system *in vivo* and will depend greatly on how much ingested toxin reaches neurons and the location of these neurons.

PSTs have been shown to be readily transported across gastrointestinal epithelium [23] and distributed throughout the body including the brain, cerebrospinal fluid, liver, bile, spleen, heart, thyroid and adrenal glands, pancreas and lungs [24]. Additionally the PSTs have been shown to have a rapid absorption with an absorption rate constant of 1.32l/h with the total amount of toxin administered being absorbed [25]. This efficient uptake and widespread distribution, as well as the ability to cross the blood brain barrier, show that a multitude of different neurons throughout the body could be affected, even at low exposure concentrations. It is unknown whether the PSTs are capable of crossing the placental barrier, potentially affecting a foetus.

Both the VGSC and calcium channels have multiple isoforms, each with different sensitivity to STX. For example there are 9 different isoforms of sodium channels ($Na_v1.1$ to $Na_v1.9$) each varying in spatial and temporal distribution in an organism and each being linked to different disease states. In humans $Na_v1.1$, $Na_v1.2$, $Na_v1.3$ and $Na_v1.6$ are found mainly in the central nervous system and $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ are found mainly in the peripheral nervous system [2]. Calcium channel isoforms include N- and P-types which are mainly neuronal and T- and L-types which are both muscular and neuronal. STX has been shown to block N-type channel activity and partially block L-type channels[3]. Therefore the adverse effects seen in vivo could vary between cell types depending on channel isoforms present. This

variation between cell types was evident here as the increase in the number of cellular projections was significantly greater in PC12 cells following exposure to 2μg/l STX than in SH-SY5Y cells. PC12 cells have been shown to express $Na_v1.2$ [26] and $Na_v1.5$ [27] whilst SH-SY5Y have been shown to express mostly $Na_v1.2$, $Na_v1.3$ and $Na_v1.7$ in addition to some $Na_v1.4$ and $Na_v1.5$ [28] and $Na_v1.9$ [29].

While the results themselves are significant and raise awareness about the possible consequences of this pattern of exposure, the underlying mechanisms causing this change in cellular morphology will still need to be determined. As mentioned above the PSTs have also been shown to act on other voltage gated channels and they have also been shown to affect antioxidant machinery [30] suggesting that the actions of the PSTs may be more complex than originally thought.

In conclusion, this work shows that extended exposure to environmentally relevant low concentrations of STX had an adverse effect on the proper development of model neuronal cells in culture. Concentrations administered were below both the shellfish and drinking water guidelines, concentrations which could very well be consumed on a daily basis, and significant effects were seen even at $1/10^{th}$ the drinking water guideline concentration. These adverse effects could have implications for the development of the nervous system as a whole. Further investigation is certainly needed into this pattern of exposure, including in vivo studies and exposure to a mix of the toxin analogues which is more likely from both sources.

2.6 Acknowledgements

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2.7 References

1. Wiese, M., et al., *Neurotoxic alkaloids: saxitoxin and its analogs.* Mar Drugs, 2010. **8**(7): p. 2185-211.

2. Savio-Galimberti, E., Gollob, M.H., and Darbar, D., *Voltage-gated sodium channels: biophysics, pharmacology, and related channelopathies.* Front Pharmacol, 2012. **3**: p. 124.

3. Jones, S.W. and Marks, T.N., *Calcium currents in bullfrog sympathetic neurons. I. Activation kinetics and pharmacology.* J Gen Physiol, 1989. **94**(1): p. 151-67.

4. Harada, T., Oshima, Y., and Yasumoto, T., *Structures of Two Paralytic Shellfish Toxins, Gonyautoxins V and VI, Isolated from a Tropical Dinoflagellate, Pyrodinium bahamense var. compressa.* Agricultural and Biological Chemistry, 1982. **46**(7): p. 1861-1864.

5. EFSA, *Marine biotoxins in shellfish - Saxitoxin group. Scientific Opinion of the Panel on Contaminants in the Food Chain.* The EFSA Journal, 2009. **1019**: p. 1-76.

6. Mahmood, N.A. and Carmichael, W.W., *Paralytic shellfish poisons produced by the freshwater cyanobacterium Aphanizomenon flos-aquae NH-5.* Toxicon, 1986. **24**(2): p. 175-186.

7. Ikawa, M., et al., *Comparison of the toxins of the blue-green alga Aphanizomenon flos-aquae with the Gonyaulax toxins.* Toxicon, 1982. **20**(4): p. 747-752.

8. Burch, M., *Effective doses, guidelines & regulations*, in *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs*, H.K. Hudnell, Editor. 2008, Springer New York. p. 831-853.

9. Campbell, X.Z. and Neet, K.E., *Hierarchical analysis of the nerve growth factor-dependent and nerve growth factor-independent differentiation signaling pathways in PC12 cells with protein kinase inhibitors.* Journal of Neuroscience Research, 1995. **42**(2): p. 207-219.

10. Hoeger, S.J., et al., *Occurrence and elimination of cyanobacterial toxins in two Australian drinking water treatment plants.* Toxicon, 2004. **43**(6): p. 639-649.

11. Jones, G.J. and Negri, A.P., *Persistence and degradation of cyanobacterial paralytic shellfish poisons (PSPs) in freshwaters.* Water Research, 1997. **31**(3): p. 525-533.

12. Markensten, H., Moore, K., and Persson, I., *Simulated lake phytoplankton composition shifts toward cyanobacteria dominance in a future warmer climate.* Ecol Appl, 2010. **20**(3): p. 752-67.

13. Paerl, H.W. and Paul, V.J., *Climate change: links to global expansion of harmful cyanobacteria.* Water Res, 2012. **46**(5): p. 1349-63.

14. Reichwaldt, E.S. and Ghadouani, A., *Effects of rainfall patterns on toxic cyanobacterial blooms in a changing climate: between simplistic scenarios and complex dynamics.* Water Res, 2012. **46**(5): p. 1372-93.

15. Brackenbury, W.J., et al., *Functional reciprocity between Na+ channel Nav1.6 and beta1 subunits in the coordinated regulation of excitability and neurite outgrowth.* Proc Natl Acad Sci U S A, 2010. **107**(5): p. 2283-8.

16. Brackenbury, W.J., et al., *Voltage-gated Na+ channel beta1 subunit-mediated neurite outgrowth requires Fyn kinase and contributes to postnatal CNS development in vivo.* J Neurosci, 2008. **28**(12): p. 3246-56.

17. Mark, M.D., et al., *Stimulation of neurite outgrowth in PC12 cells by EGF and KCl depolarization: a Ca(2+)-independent phenomenon.* J Cell Biol, 1995. **130**(3): p. 701-10.

18. Akaike, N., Furukawa, K., and Kogure, K., *Rolipram enhances the development of voltage-dependent Ca2+ current and serotonin-induced current in rat pheochromocytoma cells.* Brain Research, 1993. **620**(1): p. 58-63.

19. Jarecki, J. and Keshishian, H., *Role of neural activity during synaptogenesis in Drosophila.* The Journal of Neuroscience, 1995. **15**(12): p. 8177-8190.

20. Mattila, P.K. and Lappalainen, P., *Filopodia: molecular architecture and cellular functions.* Nat Rev Mol Cell Biol, 2008. **9**(6): p. 446-454.

21. Dent, E.W. and Gertler, F.B., *Cytoskeletal dynamics and transport in growth cone motility and axon guidance.* Neuron, 2003. **40**(2): p. 209-27.

22. Subramanian, N., et al., *Role of Na(v)1.9 in activity-dependent axon growth in motoneurons.* Hum Mol Genet, 2012. **21**(16): p. 3655-67.

23. Andrinolo, D., et al., *Transport of the organic cations gonyautoxin 2/3 epimers, a paralytic shellfish poison toxin, through the human and rat intestinal epitheliums.* Toxicon, 2002. **40**(10): p. 1389-1397.

24. Andrinolo, D., Michea, L.F., and Lagos, N., *Toxic effects, pharmacokinetics and clearance of saxitoxin, a component of paralytic shellfish poison (PSP), in cats.* Toxicon, 1999. **37**(3): p. 447-464.

25. Andrinolo, D., et al., *Toxicokinetics and toxicodynamics of gonyautoxins after an oral toxin dose in cats.* Toxicon, 2002. **40**(6): p. 699-709.

26. Fanger, G.R., et al., *Differential expression of sodium channels and nicotinic acetylcholine receptor channels in nnr variants of the PC12 pheochromocytoma cell line.* The Journal of Membrane Biology, 1995. **144**(1): p. 71-80.

27. Safo, P., et al., *Distinction among Neuronal Subtypes of Voltage-Activated Sodium Channels by Œº-Conotoxin PIIIA.* The Journal of Neuroscience, 2000. **20**(1): p. 76-80.

28. Vetter, I., et al., *Characterisation of Nav types endogenously expressed in human SH-SY5Y neuroblastoma cells.* Biochemical Pharmacology, 2012. **83**(11): p. 1562-1571.

29. Blum, R., Kafitz, K.W., and Konnerth, A., *Neurotrophin-evoked depolarization requires the sodium channel Na(V)1.9.* Nature, 2002. **419**(6908): p. 687-93.

30. Ramos, P.B., et al., *Oxidative stress in rats induced by consumption of saxitoxin contaminated drink water.* Harmful Algae, 2014. **37**(0): p. 68-74.

3. Optimization of a real time resazurin based assay for use in OVCAR-3 and SH-SY5Y cells

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Contribution to the Paper	Performed all experimental work, interpreted data, wrote manuscript and acted as corresponding author.
Overall percentage (%)	
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 26/5/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate to include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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3.1 Abstract

Assays which can accurately and reliably assess the anti-proliferative or cytotoxic activity of test compounds are essential to pharmaceutical drug design and toxicology. Ideally an assay would be cost-effective, high-throughput, non-hazardous to users and would allow repeated measurements using non-destructive assessment methods. Many assay exist but each have their drawbacks and none deliver each of these described characteristics. One such assay that may meet these requirements is a colourmetric resazurin based assay. Resazurin indicates viability indirectly through cellular metabolism. Here we describe an expanded and optimized resazurin based assay previously described using the OVCAR-3 cell line. OVCAR-3 cells were exposed to resazurin (50-700mM) for 1-72h and SH-SY5Y cells exposed to resazurin (100mM) for 1-64h with absorbance read on the same cells at multiple times during the exposure period. The toxicity of resazurin was assessed at 16, 40 and 64h with a crystal violet assay. Linear ranges of dye reduction were determined in each cell line. Resazurin was not toxic at 16h, but caused a significant reduction in cell viability following 40h. Following optimisation the assay was tested with SH-SY5Y cells. Cells were exposed to STX (0.25-10μg/L) or ZnSO4 (10^{-4}-10^{-1}M) in resazurin (100µM) containing medium with absorbance read at 0, 12 and 24h. The assay correctly identified the concentration dependent cytotoxic effect of $ZnSO_4$, but no change in reduction was seen following exposure to STX. We have shown that following proper optimization a resazurin-based assay can be used for a continuous monitoring of cell viability, indirectly through cellular metabolism.

3.2 Introduction

Assays which can accurately and reliably assess the anti-proliferative or cytotoxic activity of test compounds are essential to pharmaceutical drug design and toxicology. Ideally an assay would be cost-effective, high-throughput, non-hazardous to users and would allow repeated measurements using non-destructive assessment methods.

Many assays are available but none deliver all of these appealing characteristics. The commonly used MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], crystal violet, ATP bioluminescence, ^{3}H-thymidine incorporation and lactate dehydrogenase (LDH) leakage assays all have endpoints that require cell death so that multiple assessments cannot be made on the same culture plates [1, 2]. As a result, a separate cell culture plate is required for each time point which increases costs of labor and reagents. These assays also have multistep protocols that increase the duration of the assay and the ^{3}H-thymidine incorporation assay requires the use of a radioactive compound. A non-destructive continuous assay which allowed the assessment of viable cell numbers at consecutive time points would be more cost effective and also generate invaluable data regarding the half-life or stability of test compounds *in vitro*.

Further, in these above mentioned assays multiple steps are required. Having a multistep protocol not only adds to time required to carry out an assay it also increases the chances of variation between repeats. For example the crystal violet assay requires a rinsing step which is vulnerable to variation as has been recorded previously [3].

Compared to other metabolic assays such as the MTT or lactate dehydrogenase leakage assay (LDH), which only measure the activity of one enzyme, a resazurin based assay measures the activity of many enzymes facilitating a comprehensive assessment of integrated metabolic activity of the cell, which may be a more informative measurement of overall cell status and viability than a single enzyme.

Cytotoxicity assays are based on different mechanisms of action. Crystal violet (Tris (4-(dimethylamino) phenyl) methylium chloride) stains DNA [4] and has been described as a stain for cell nuclei [5, 6]. As such, the crystal violet assay measures the number of viable cells rather than their metabolic activity. Rothman [7] reported using crystal violet to quantify HeLa cells after exposure to toxins, and Gillies et al [3] presented a crystal violet assay with a linear correlation between cell number and optical density. Kueng et al [6] introduced 10% acetic acid as a destain and also described a standard curve. Different versions of a crystal violet cytotoxicity assay have compared well with other methods for assessing cytotoxicity [8, 9], although cell lines with high proliferation rates can have an increased proportion of binucleate cells, which result in over-estimates in a crystal violet assay [10]. A single crystal violet assay protocol applied in 14 different laboratories was highly reproducible [11].

In the Trypan Blue exclusion assay viable cells possess intact cell membranes which exclude the dye, whereas dead or damaged cells lacking an intact plasma membrane are stained blue. A cell suspension is mixed with

the Trypan Blue dye and examined using a microscope and hemocytometer to determine the number of viable cells per milliliter.

The Alamar Blue (AB) assay is a cheap, one step assay which is non-toxic to users, high-throughput, does not require cell death and has shown high reproducibility [2, 12]. The active compound of AB, blue resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one), is taken up by viable cells and irreversibly converted to pink resorufin which is then excreted to the culture media causing a color change [13]. Resazurin is an intermediate electron acceptor reduced by numerous enzymes within the cell, mostly mitochondrial and cytosolic as well as microsomal [2, 14], without interfering with normal electron transport [2]. Hence resazurin reduction is a measure of general cellular metabolic status and not of any single enzyme. The reduction of the dye can be measured quantitatively by either colormetric or fluorometric readings. The fluorescent signal can be quenched by proteins in culture media [15] so although measuring fluorescence may be more sensitive, measuring absorbance avoids interference.

Exposing cells to resazurin for extended periods of time however can result in secondary reduction of resorufin to colorless dihydroresorufin [2] and prolonged exposure to resazurin has been shown to be toxic. Exposure to resazurin for 6h reduced cell proliferation and caused mitochondrial changes and initiation of apoptosis after 24h [16]. The extent of these changes depended on the cell line and were thought to be a result of increased reactive oxygen species (ROS) production [16], exposure and concentration of AB or resazurin. The toxicity of resazurin after extended periods in culture

may give false positives for toxicological screening of test compounds. Hence there is a need to define concentration and exposure regimes which are not cytotoxic.

The reduction of resazurin to resorufin is dependent upon the metabolic rate of the cell line [17], and perhaps also upon the resistance of the cell line to the cytotoxic effects of the dye. The metabolic status of a cell line is related to proliferation rate, which in turn can be related to cell density and for some cell lines low cell density is associated with low proliferation rates and low resorufin production [2]. It has been recommended that cell lines should be in the exponential growth phase, and should be incubated with at least three concentrations of resazurin at various cell densities and incubation times to determine optimal resazurin assay conditions [17].

When AB was used to examine the ovarian OVCAR-3 cell line the linear range was 0.05 - $2x10^4$ cells per well in a 96 well plate, with an AB concentration of 4% v/v in a 3h exposure [18]. The Molar concentration of resazurin in the AB was not stated, but in a separate study the concentration of resazurin in AB was determined to be 400μM [13], which suggests that the OVCAR-3 study used a resazurin concentration of approximately 16μM. This second study however used AB from a different source.

The conditions used to examine the OVCAR-3 cell line [18] were repeated and extended in the present study, and were additionally applied to the neuronal SH-SY5Y cell line, to develop a real time assay which could later be used to monitor the effects of test compounds on cellular proliferation and metabolism. We aimed to determine resazurin concentrations and exposure

times which did not significantly affect cell viability. Since resazurin reduction is dependent on the metabolism and proliferation rate of a cell line, we examined and compared the proliferation rate of the cell lines using resazurin, the Trypan Blue exclusion assay, and a crystal violet assay.

Following optimization of the assay, it was tested using the SH-SY5Y cell line exposed to $ZnSO_4$, a substance known to induce apoptosis through various pathways [19], and Saxitoxin (STX), a substance which blocks voltage gated sodium channels and disrupts the morphology of SH-SY5Y cells in culture but does not cause cytotoxicity [20] . Using the 3 assays we aimed to distinguish between effects on metabolism, cytotoxicity and proliferation rate in these cells.

3.3 Methods

3.3.1 Cell culture

Human ovarian adenocarcinoma cells, OVCAR-3, and human neuroblastoma cells, SH-SY5Y, were obtained from the ATCC. The OVCAR-3 cell line was maintained in Roswell Park Memorial Institute (RPMI) 1640 with sodium bicarbonate (1.5g/L), HEPES (10mM), 1mM sodium pyruvate (0.11g/L), penicillin/streptomycin (5000µg/ml), glucose (4.5g/L), L-glutamine (2mM), insulin (0.01mg/ml) and 20% heat inactivated fetal bovine serum. The SH-SY5Y cell line was maintained in Dulbecco's Modified Eagle Media (DMEM) supplemented with 5% fetal bovine serum, 200mM L-Glutamine, 1000U/ml penicillin and streptomycin and 1% non-essential amino acids. Cells were cultured at 37^{o}C with 5% CO_2 and passaged every 3-4 days.

3.3.2 Determining optimal seeding density, exposure time and resazurin concentration

OVCAR-3 cells ($1.6x10^5$ cells per well) were added to 6 replicate wells of ten 96 well plates in 200µl media and 100µl media were added to the remaining wells. Cells were serially diluted 1:2 giving a gradient of 0 to $8x10^4$ cells per well in 100µl media. This cell line was allowed to adhere for 24h after which time media were aspirated from 5 plates and replaced with 200µl media containing a different concentration of resazurin per plate (50, 175, 350 and 700µM). One hour after the addition of resazurin the plate containing 50µM resazurin was sealed with a sterile plate sealer and absorbance was read at 570 and 600nm. The remaining plates with different concentrations of resazurin were first read at 2h. Absorbance was read again after 3, 4, 5, 6, 24 and 48h for the 50µM resazurin plate and after 4, 6, 24, 48 and 72h for 175, 350 and 700µM plates. The remaining plates (no resazurin) were assessed using the crystal violet assay after 2, 4, 6, 24, 48 and 72h. The experiment was repeated on three separate occasions (n=3).

SH-SY5Y cells were seeded into six 96 well plates in the same manner as the OVCAR-3 cells, to generate a gradient of 0 to $8x10^4$ cells per well in 100µl media. SH-SY5Y cells were allowed to adhere for 6h after which time media were aspirated from 3 plates and replaced with 200µl media containing 100µM resazurin. One hour after the addition of resazurin 1 plate was sealed with a sterile plate sealer and absorbance was read at 570 and 600nm. The plate sealer was removed and the cells were returned to incubation. Absorbance was read on the same plate again after 16, 18, 20, 22, 24, 26, 40, 42, 44, 46, 48, 50 and 64h. At 16, 40 and 64h two of the 5

replicate plates had media (one plate contained resazurin and the other media only) removed. The cells were washed before completing the crystal violet viability assay (see below) to determine the cytotoxicity of resazurin. The experiment was repeated on three separate occasions (n=3).

3.3.3 Comparison of MTT and resazurin based assays for detecting OVCAR-3 viability

OVCAR-3 cells ($1.6x10^5$ cells per well) were added to 6 replicate wells of one 96 well plates in 200µl media and 100µl media were added to the remaining wells. Cells were serially diluted 1:2 giving a gradient of 0 to $8x10^4$ cells per well in 100µl media. OVCAR-3 cells were additionally seeded at $2x10^4$ cells/well in four 96 well plates. After 24h the serially diluted plate was subjected to MTT assay and a standard curve was created. Remaining plates had medium removed and replaced with medium alone, medium containing 1% DMSO or medium containing 0.1g/ml H_2O_2, two plates additionally received 50μM resazurin. At 4 and 48h one plate without resazurin was subjected to the MTT assay and one with resazurin had absorbance read as described above. Absorbance values were converted to viable cell numbers using MTT standard curve and resazurin standard curves. The experiment was repeated on three separate occasions (n=3).

3.3.4 Crystal violet assay

Media were removed from all wells and replaced with 50µl of 0.5% crystal violet in 50% methanol. After 10 minutes crystal violet was removed and cells

were gently washed with running water. Plates were left to dry overnight, after which time 50µl of 33% acetic acid was added and incubated at room temperature for 10 minutes. Absorbance was read at 570nm with 630nm correction.

3.3.5 MTT assay

Media were removed from all wells, replaced with 100μl of 0.5mg/ml MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) and incubated at 37^{o}C with 5% CO_2. At 18h 80μl of 20% SDS in 0.02 HCl was added to each well and incubated at room temperature for 1h in the dark. Absorbance was read at 570nm with reference correction 630nm.

3.3.6 Proliferation and doubling time of SH-SY5Y cells

SH-SY5Y cells were added to 3 wells of 5 replicate 6 well plates at 3 different densities (1, 2.5 and 5x10^{4} cells per well) in 2mL of media. Cell numbers were determined at 24hr intervals until 120h. Media were not replenished to allow comparison with the resazurin based assay where media replenishment is not possible. At the end of each 24hr interval, cells in one replicate plate were trypsinised and counted using the Trypan Blue exclusion assay. Briefly, 20μl of cell suspension was added to a 1.5ml Eppendorf tube and diluted with 20μl trypan blue, 10μl of the mixture is added to a hemocytometer. Using a microscope cells were viewed and the number of living cells (those not dyed blue) in the four 4x4 squares are counted.

The number of viable cells per ml was determined using equation 3.1 below.

$$\text{Cells/ml} = (\text{cell count} / 4) \times 2 \times 10^4$$

Equation 3.1

These data were used to determine the doubling time using equation 3.2 below where *t* equaled time of incubation, N_0 equaled initial number of cells and N_t equaled final number of cells. The experiment was repeated on three separate occasions (n=3).

$$\text{Doubling time } (Td) = t \times (\log 2 / \log 2 (N_t/N_0))$$

Equation 3.2

3.3.7 Effect of $ZnSO_4$ and STX on SH-SY5Y metabolism using resazurin

SH-SY5Y cells were seeded at a density of $2x10^4$ cells/well into two 96 well plates in 100μl media. Cells were allowed to adhere for 6h after which time media was removed and replaced with 200μl media alone, 200μl media containing 100μM resazurin, 200μl media containing 100μM resazurin and $ZnSO_4$ (10^{-4}-10^{-1}M) or 200μl media containing 100μM resazurin and STX (0.25-10μg/L). Immediately following addition of treatments one plate was sealed with a sterile plate sealer and absorbance was read at 560 and 600nm, before removing the plate sealer and being returned to incubation. Absorbance was read again at 12 and 24h. The experiment was repeated on three separate occasions (n=3).

3.3.8 Data analysis

Intra- and inter-assay co-efficient of variability (%CV) were calculated for the SH-SY5Y resazurin assay. The intra-assay %CV was determined by calculating the standard deviation of technical replicates, dividing that by the mean of the technical replicates and multiplying by 100. The %CVs for each density were then used to determine the average intra-assay %CV. The inter-assay %CV was determined by calculating the standard deviation of experimental means, dividing by the mean of these, and multiplying by 100. The %CVs for each density were then used to determine the average inter-assay %CV.

The cytotoxicity of resazurin to SH-SY5Y cells and viability of OVCAR-3 cells following exposure to DMSO or H_2O_2 was statistically analyzed using two-way ANOVA with Sidak's multiple comparisons test ($p \leq 0.05$). Reduction of resazurin by OVCAR-3 and SH-SY5Y cells, proliferation of SH-SY5Y cells and $ZnSO_4$ and STX toxicity was statistically analyzed using two-way ANOVA with Tukey's multiple comparisons test ($p \leq 0.05$). Comparison of MTT and resazurin assay were analyzed using one-way ANOVA with Dunn's multiple comparisons test ($p \leq 0.05$).

3.4 Results

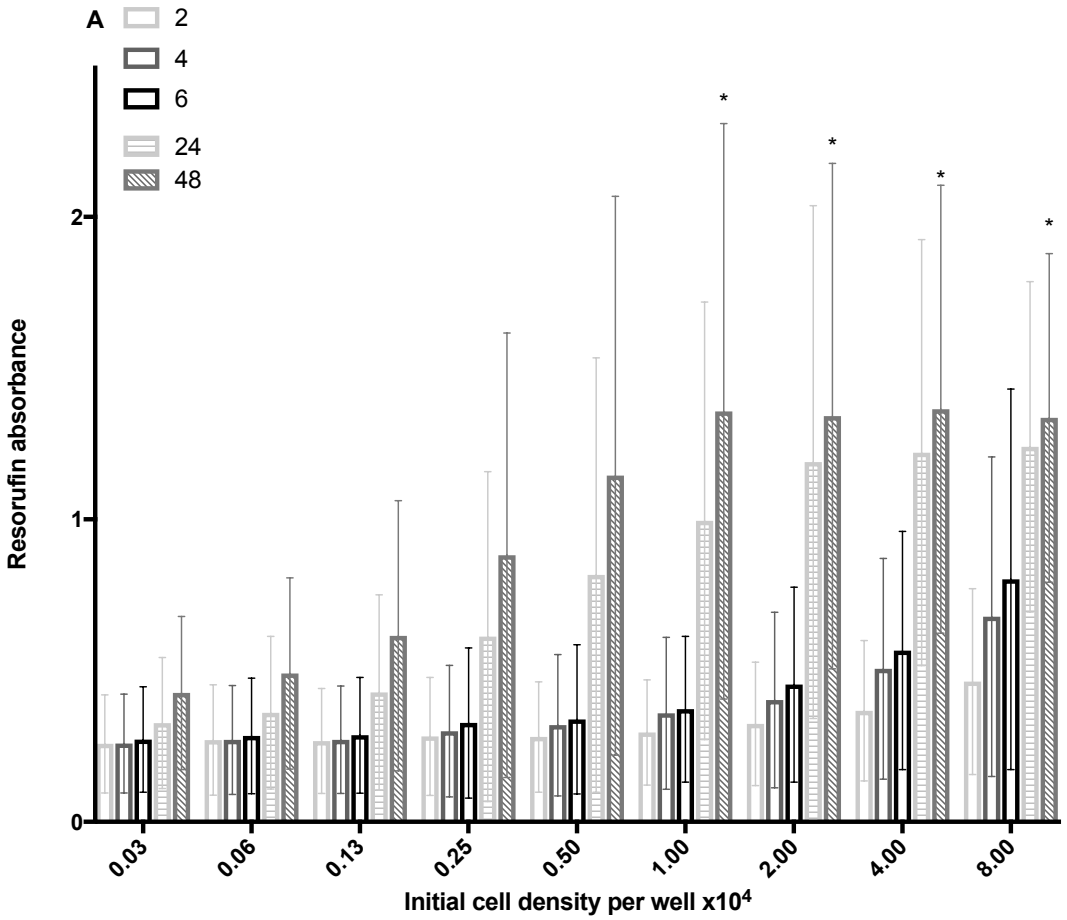
3.4.1 Effect of cell density, exposure time and resazurin concentration on resorufin production by OVCAR-3 cells

In cultures exposed to 50μM resazurin there was a cell density and time dependent linear increase in resorufin production in the first 6h of culture for a broad range of cell densities (0.06-8x10^4cells/well), but linearity was

reduced for longer incubation times. Significant increases in resorufin production compared to initial 2h measurements were seen for seeding densities above $1x10^4$ cells/well (Figure 3.1A, select times shown).

This linearity decreased as the concentration of resazurin increased making it more difficult to differentiate between the higher cell densities when cells were cultured with 175μM or 300μM resazurin for the same periods of time (Figure 3.1B-C). Further when seeding density exceeded $2x10^4$cells/well, resorufin production was not proportional to time in culture for longer than 24h. Significant increases in resorufin production were seen at all densities compared to the initial 2h measurement for these 2 midrange concentrations (Figure 3.1B-C, select times shown).

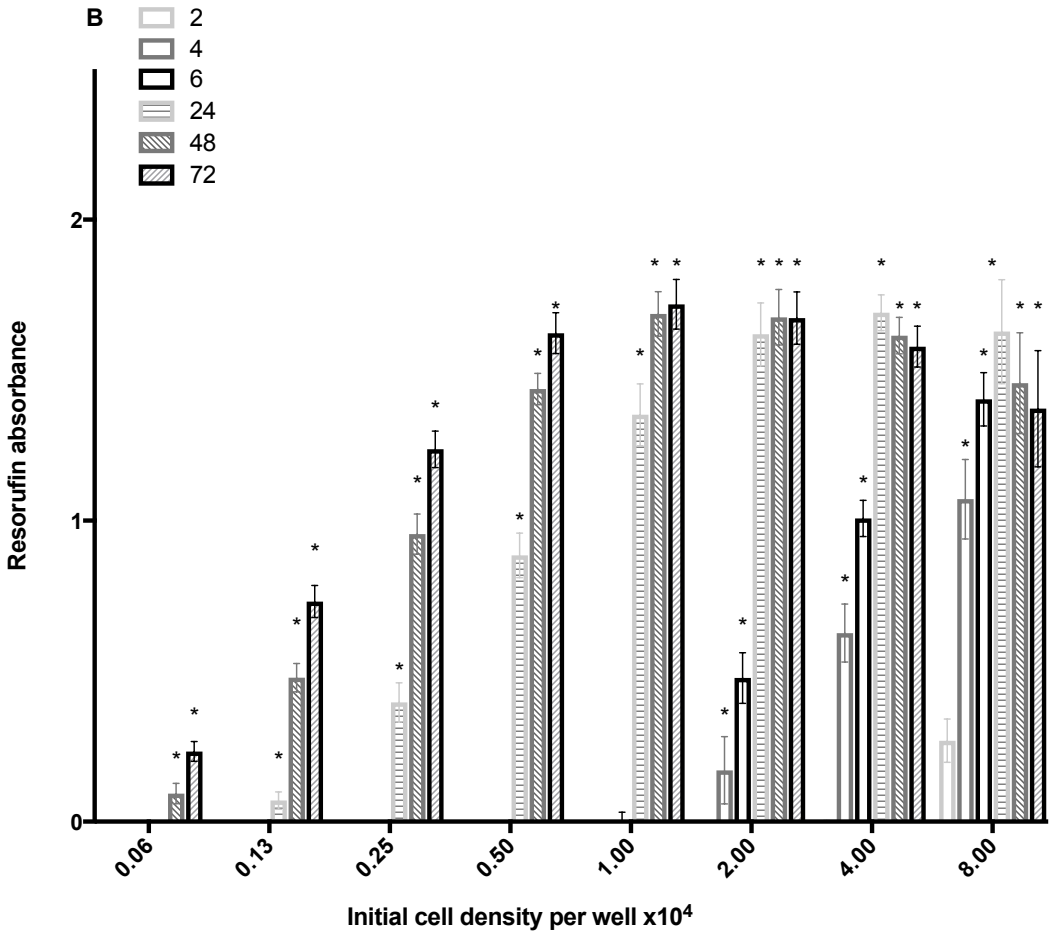
A resazurin concentration of 700μM did not support the relationship between cell density and resorufin production (Fig 3.1D). The rate at which resorufin was produced was reduced at the two highest concentrations but similar maximum ODs were still reached. Significant increases in resorufin production were seen at cell densities greater than $0.25x10^4$ compared to the initial 2h measurement for this highest resazurin concentrations (Fig 3.1D, select times shown).



Initial cell density per well x10 $^{-4}$	0.03	0.06	0.13	0.25	0.5	1	2	4	8
R^2	0.9906	0.9909	0.8802	0.9937	0.9824	0.9739	0.9108	0.9017	0.8156
P value	<0.0001	<0.0001	0.0006	<0.0001	<0.0001	<0.0001	0.0002	0.0003	0.0021

Figure 3.1A Reduction of resazurin by OVCAR-3 cells.

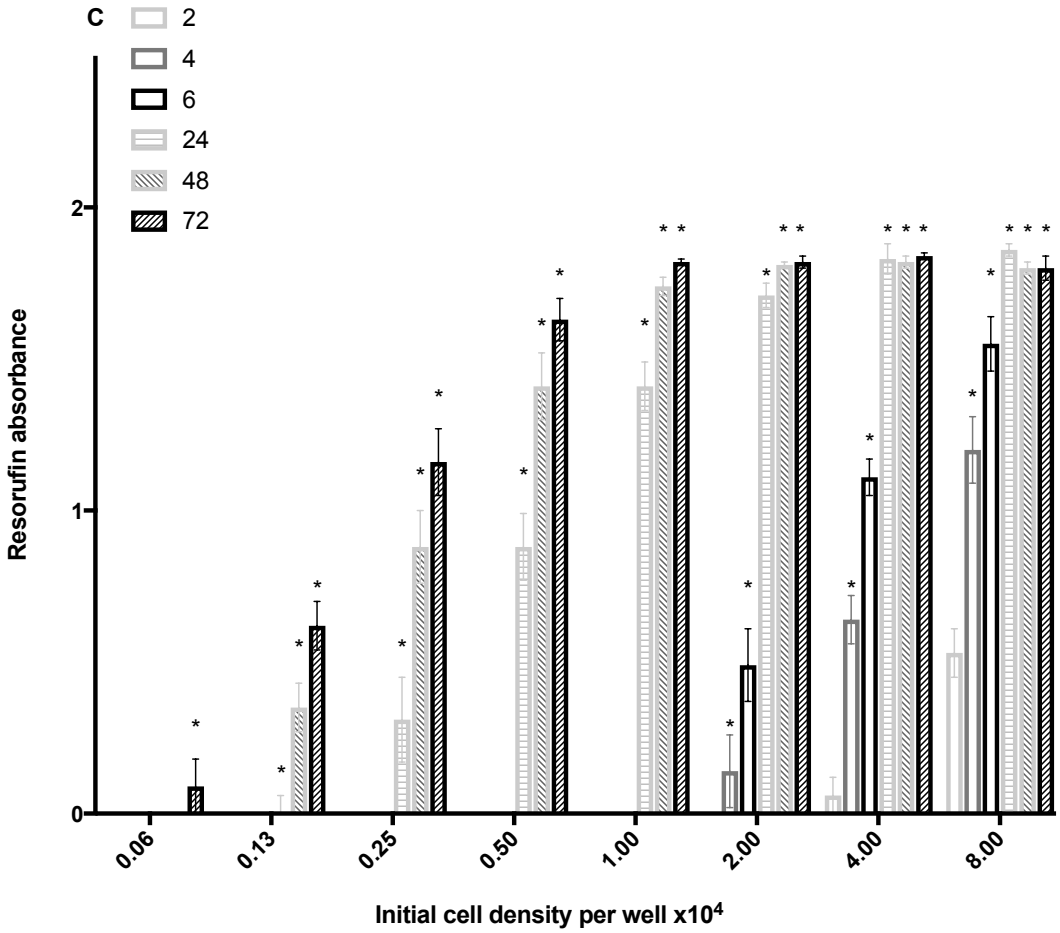
*Cells were plated in 96 well plates and exposed to resazurin at 50μM (**A**) (for clarity only select times shown), 175μM (**B**), 300μM (**C**) 700μM (**D**) for 1-72h before absorbance was read. R2 and P values calculated using linear regression for each initial cell density over time. Data is expressed as mean absorbance minus background ± SD (n=3) and was subjected to two-way ANOVA with Tukey's multiple comparisons test. *p<0.05 compared to initial 2h reading for same density.*



Initial cell density per well x10 ^{4}	0.06	0.13	0.25	0.5	1	2	4	8
R^2	0.9477	0.9634	0.9488	0.8969	0.8066	0.6967	0.533	0.2364
P value	0.001	0.0005	0.001	0.0041	0.0151	0.0387	0.0995	0.3281

Figure 3.2B Reduction of resazurin by OVCAR-3 cells.

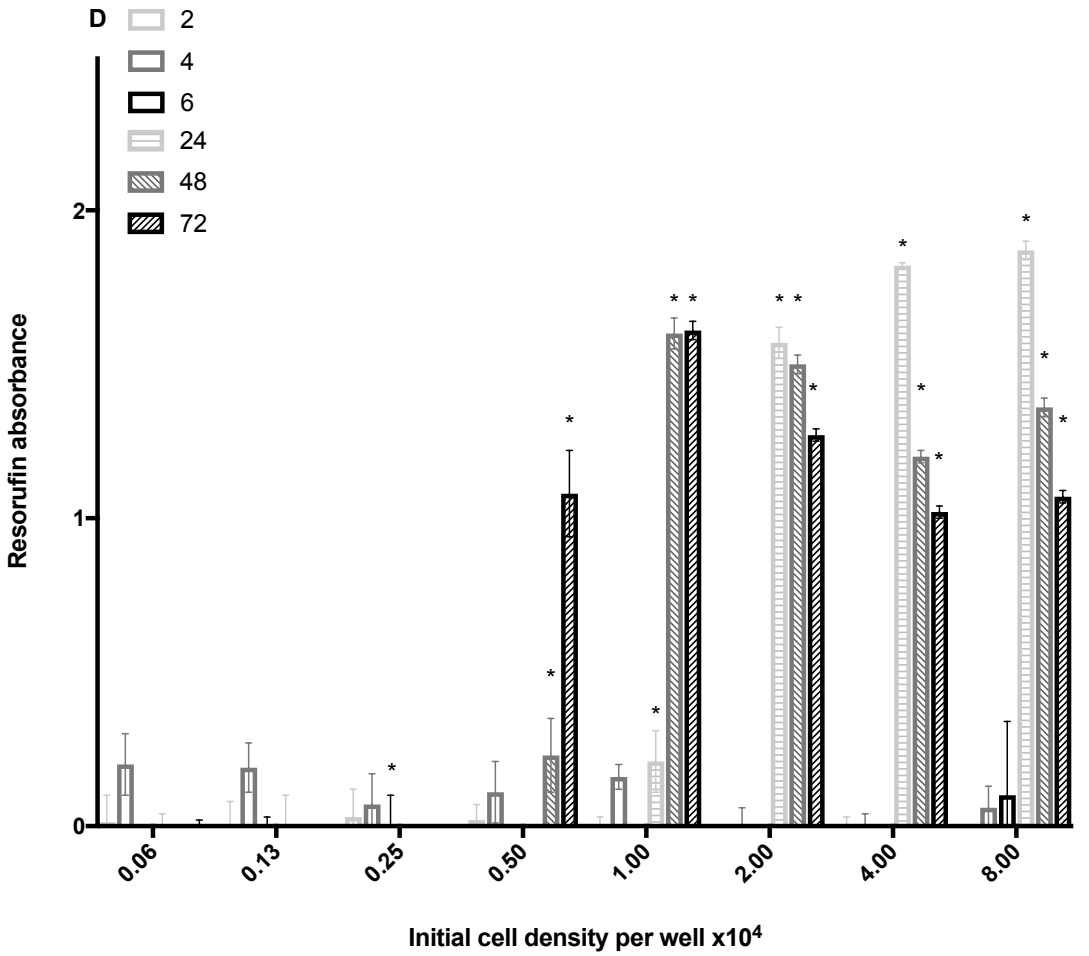
*Cells were plated in 96 well plates and exposed to resazurin at 50μM (**A**) (for clarity only select times shown), 175μM (**B**), 300μM (**C**) 700μM (**D**) for 1-72h before absorbance was read. R2 and P values calculated using linear regression for each initial cell density over time. Data is expressed as mean absorbance minus background ± SD (n=3) and was subjected to two-way ANOVA with Tukey's multiple comparisons test. *p<0.05 compared to initial 2h reading for same density.*



Initial cell density per well x10 ^{4}	0.06	0.13	0.25	0.5	1	2	4	8
R^2	0.9497	0.9439	0.9398	0.8919	0.8148	0.7219	0.6075	0.4357
P value	0.001	0.0012	0.0014	0.0046	0.0138	0.0322	0.0676	0.1537

Figure 3.3C Reduction of resazurin by OVCAR-3 cells.

*Cells were plated in 96 well plates and exposed to resazurin at 50μM (**A**) (for clarity only select times shown), 175μM (**B**), 300μM (**C**) 700μM (**D**) for 1-72h before absorbance was read. R2 and P values calculated using linear regression for each initial cell density over time. Data is expressed as mean absorbance minus background ± SD (n=3) and was subjected to two-way ANOVA with Tukey's multiple comparisons test. *p<0.05 compared to initial 2h reading for same density.*



Initial cell density per well x10 ^{4}	0.06	0.13	0.25	0.5	1	2	4	8
R^2	0.3542	0.4242	0.3639	0.709	0.8838	0.6034	0.399	0.4072
P value	0.2127	0.1612	0.2049	0.0355	0.0053	0.0692	0.1785	0.1727

Figure 3.4D Reduction of resazurin by OVCAR-3 cells.

*Cells were plated in 96 well plates and exposed to resazurin at 50μM (**A**) (for clarity only select times shown), 175μM (**B**), 300μM (**C**) 700μM (**D**) for 1-72h before absorbance was read. R2 and P values calculated using linear regression for each initial cell density over time. Data is expressed as mean absorbance minus background ± SD (n=3) and was subjected to two-way ANOVA with Tukey's multiple comparisons test. *p<0.05 compared to initial 2h reading for same density.*

3.4.2 OVCAR-3 viability over exposure period

The measurement of OVCAR-3 viability was compared using MTT and resazurin assays over 48h (Fig 3.2). The MTT and resazurin assays gave consistent results; exposure to 1% DMSO did not affect viability at either 4 or 48h, whereas viability was significantly reduced increasingly from 4 to 48h after exposure to H_2O_2 (Fig 3.2). There was no significant difference between assay types for each treatment group.

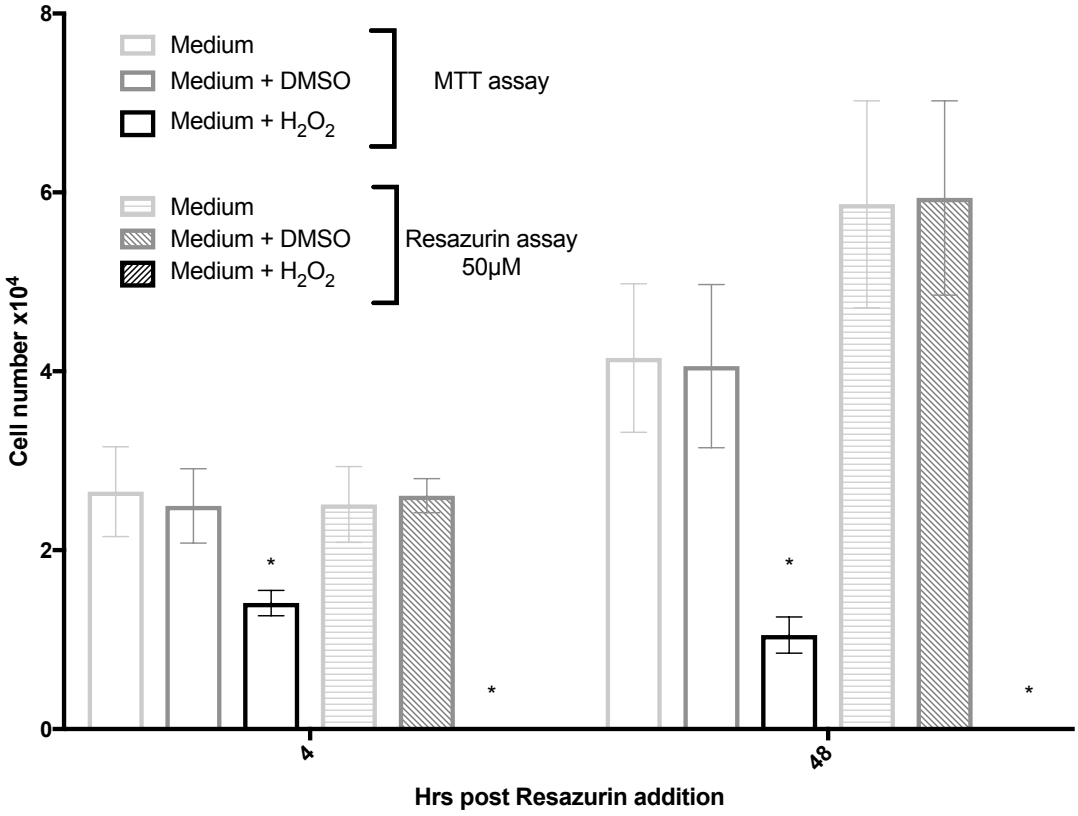


Figure 3.5 Comparison of MTT and resazurin assays for detecting OVCAR-3 viability.

*Cells were plated in 96 well plates at 2 x10^4 cells/well and exposed to medium alone (light grey bars), medium with 1% DMSO (dark grey bars) or medium with 0.1g/ml H_2O_2 (black bars) and assessed using the resazurin assay (patterned bars) or MTT assay (empty bars) after 4 and 48h. Optical density values were converted to cell number by comparison with a standard curve and data expressed as average cell number ± SD (n=3) Data were subjected to two-way ANOVA with Sidak's multiple comparisons test. *p<0.05 compared to medium with 0.1g/ml H_2O_2 at same exposure.*

3.4.3 Doubling time of SH-SY5Y cells

Proliferation was supported for the first 96h of culture with the largest increase in cell numbers seen between 48-72h and 72-96h for the initial seeding densities of $5x10^4$ cells/well and $2.5x10^4$ cells/well respectively (Fig 3.3). Following 96h cell numbers for these 2 higher densities decreased likely due to nutrient depletion and/or space limitations. Doubling times were fastest between 24-48h for the two larger seeding densities correlating to larger percentage increases in cell numbers, which slowed over time (Table 3.1).

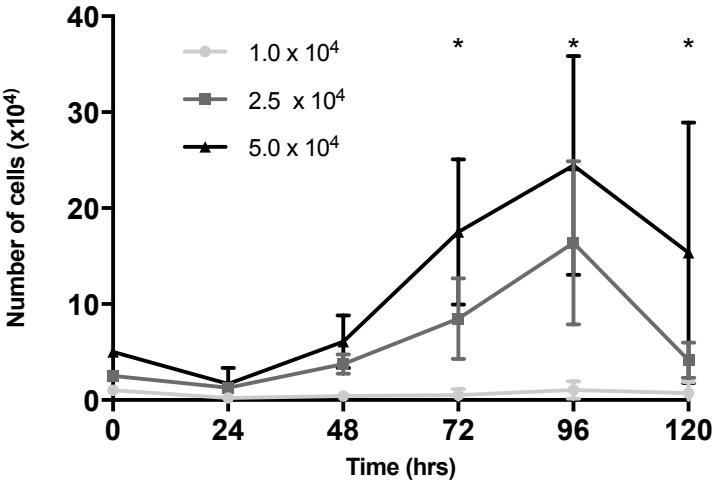


Figure 3.6 Proliferation of SH-SY5Y cells.

*Cells were plated in 6 well plates at $1x10^4$ cells/well (light grey circles), $2.5x10^4$ cells/well (dark grey squares) and $5x10^4$ cells/well (black triangles). Viable cell numbers were counted using the trypan blue assay every 24h for 120h. Data is expressed as mean cell counts ± SD (n=3) and was subjected to two-way ANOVA with Tukey's multiple comparisons test. *$p<0.05$ $1x10^4$ cells/well compared to all densities at 72h and 96h and 5 $x10^4$ cells/well compared to all densities at 120h.*

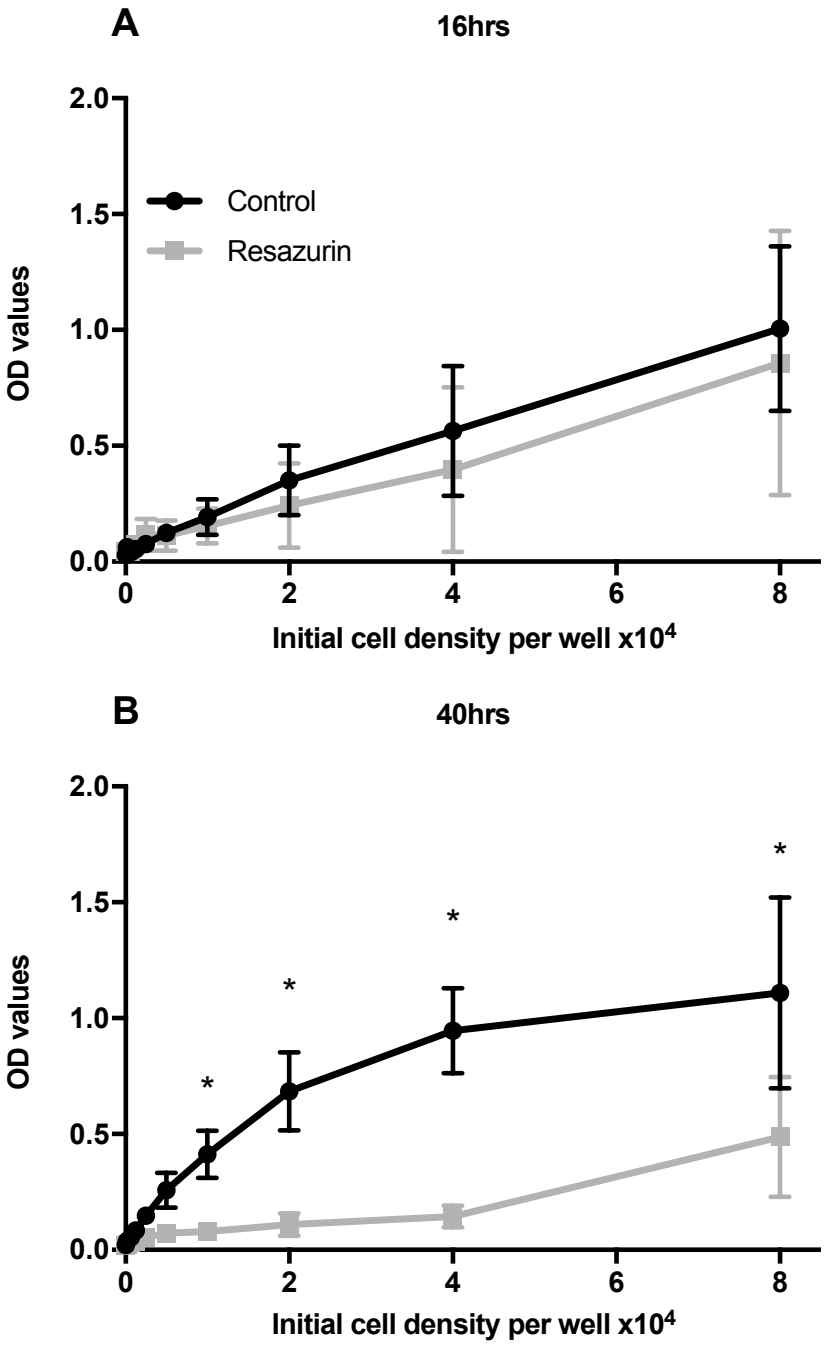
Seeding density per well	24-48h	48-72h	72-96h
$1x10^4$	26	76	23
$2.5x10^4$	16	20	25
$5x10^4$	13	16	50

Table 3.1 Doubling times (h) of SH-SY5Y cells.

Cells were plated in 6 well plates at $1x10^4$ cells/well, $2.5x10^4$ cells/well and $5x10^4$ cells/well. Viable cell numbers were counted using the trypan blue assay every 24h for 120h and doubling times were calculated from mean cell counts (n=3) for 24h periods for 24-96h.

3.4.4 Resazurin cytotoxicity in SH-SY5Y cells

The crystal violet viability assay was conducted after incubation for 16, 40 and 64h in the presence or absence of resazurin. Toxicity was not significant in the presence of 100μM resazurin at 16h (Fig 3.4). However, following 40h control cells at all seeding densities continued to proliferate while cells exposed to resazurin had significantly lower crystal violet staining. Similarly; control cell numbers continued to grow over 64h for initial densities fewer than $2x10^4$ cells/well but for high seeding densities viable cell numbers decreased between 40 and 64h suggesting limitations on nutrition or space. Staining at 64h for cells exposed to resazurin was significantly lower than the relevant control when initial seeding densities were greater than $1x10^4$ cells/well.


A
16hrs
2.0
1.5
1.0
0.5
0.0
OD values
Control
Resazurin
0
2
4
6
8
Initial cell density per well x10^4
B
40hrs
2.0
1.5
1.0
0.5
0.0
OD values
*
*
*
*
0
2
4
6
8
Initial cell density per well x10^4

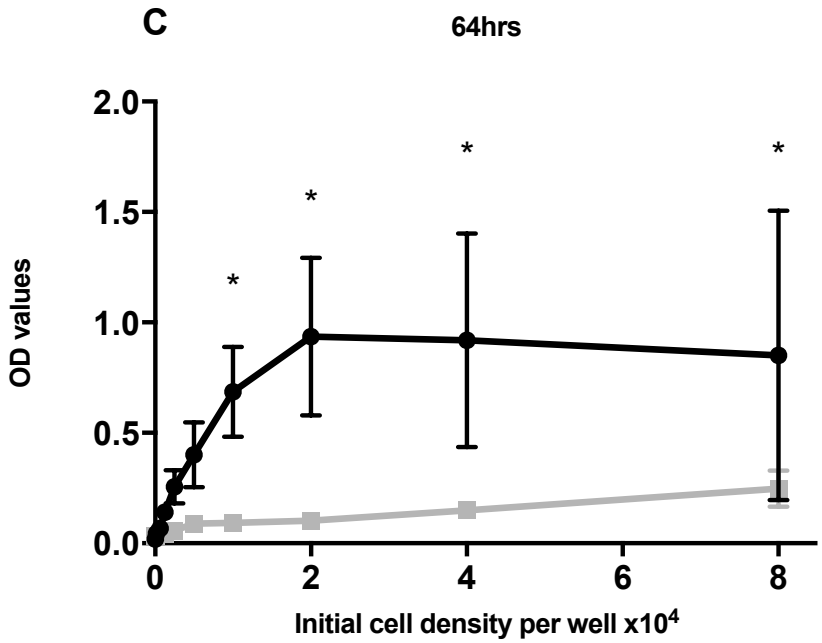


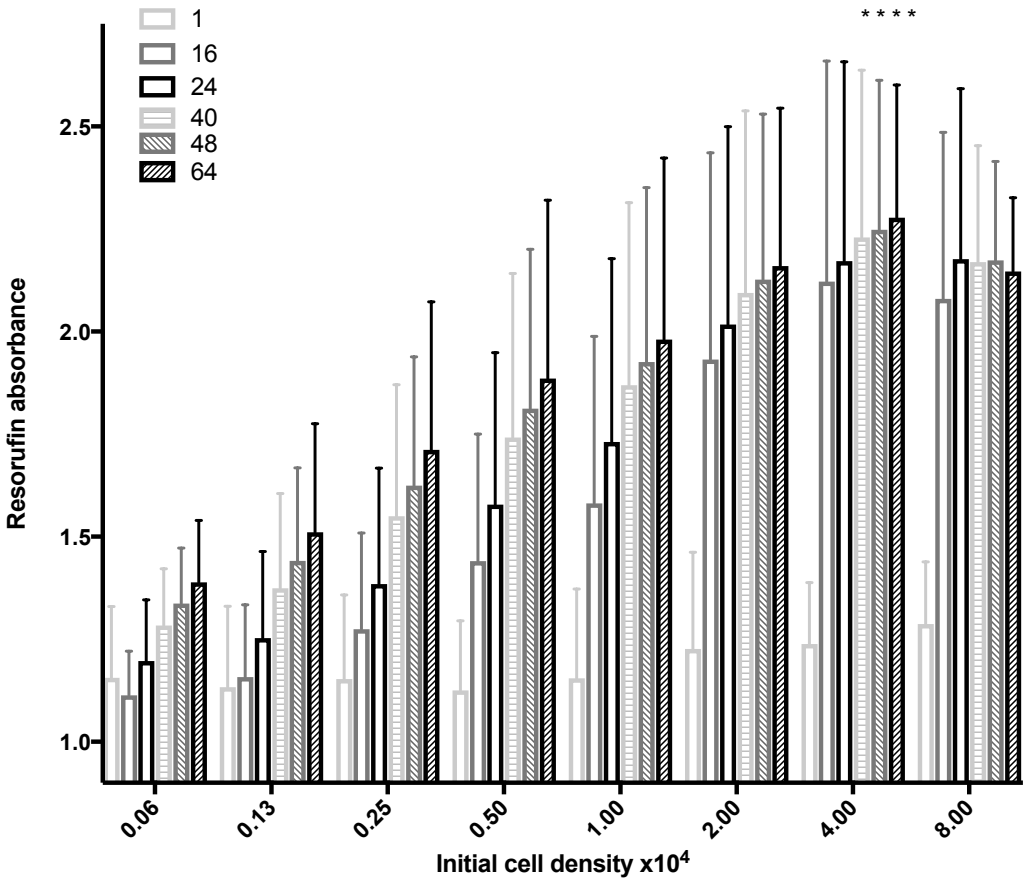
Figure 3.7 Effect of resazurin on SH-SY5Y cell viability.

*Cells were plated in 96 well plates and exposed to resazurin (100μM, black circles) or medium alone (grey squares) for 16, 40 and 64h before undergoing crystal violet assay. Data is expressed as mean absorbance ±SD (n=3) and was subjected to two-way ANOVA with Sidak's multiple comparisons test. *p<0.05 resazurin exposure compared to medium alone for same time point.*

3.4.5 Effect of cell density and exposure time on resazurin reduction by SH-SY5Y cells

To examine the effect of cell seeding density and exposure time on the reduction of resazurin, resorufin production was measured over a 64h period at various initial cell densities (Fig 3.5, select times). The increases in resorufin production were seeding cell-density and time dependent, but the increases were linear with respect to initial cell density in the range of 0.13 to $0.5x10^4$ cells / well (Fig 3.5). There was a significant difference in resorufin measured at 64h compared to the initial measurement at 1h for wells with an initial cell density of 4×10^4. The largest differences between resorufin values at different time points (best discrimination) occurred with a seeding density

of 0.5 x 10^4 cells/well but these differences did not reach statistical significance. The intra-assay variation was in the order of 2.3-4.3% and inter-assay variation was in the order of 16.3-20.7%



Initial cell density per well x10 ^{4}	0.06	0.13	0.25	0.5	1	2	4	8
R^2	0.8961	0.9694	0.9869	0.9355	0.8469	0.5789	0.4378	0.3379
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0016	0.01	0.0292

Figure 3.8 Reduction of resazurin by SH-SY5Y cells.

*Cells were plated in 96 well plates and exposed to resazurin (100μM) for 1-64h (for clarity, only select times shown) before absorbance was read. R2 and P values calculated using linear regression for each initial cell density over time. Data is expressed as mean absorbance ± SD (n=3) and was subjected to two-way ANOVA with Tukey's multiple comparisons test. *p<0.05 compared to initial 1h reading for same density.*

3.4.6 Effect of $ZnSO_4$ and STX on SH-SY5Y metabolism using resazurin

SH-SY5Y cells ($2x10^4$ per well) that had adhered for 6h, then had 100μM resazurin added, produced a background OD value of 1.2 ± 0.05 with no time to produce resorufin at 0h. After 12h incubation with resazurin, the OD value significantly increased to 2.3 ± 0.1 but an additional 12h incubation did not result in a significant increase in resorufin. The 0h OD values were the same as controls after the addition of $ZnSO_4$ or STX to resazurin indicating that they did not affect the OD or interfere with the assay. $ZnSO_4$ concentration dependently decreased resorufin production following 12 and 24h, with all concentrations resulting in significantly lower OD values compared to controls for the same time (Fig 3.6A).

Following 12h exposure to STX there was no change in resorufin production with a similar increase in OD value for all concentrations and controls (Figure 3.6B). A small non-significant increase was seen at 24h with the exception of 4μg/L and 8μg/L STX where OD values were significantly lower.

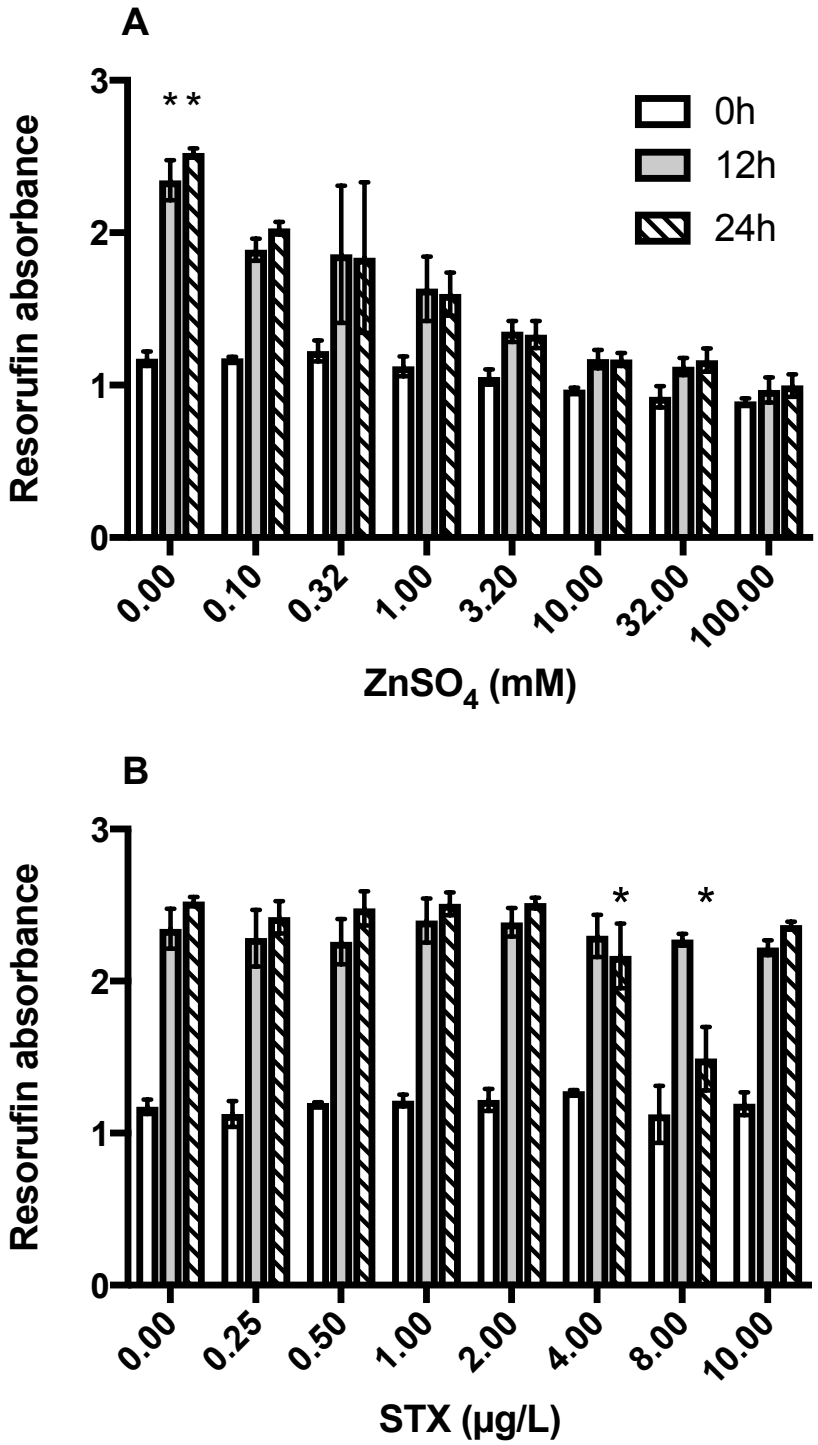


Figure 3.9 Effect of ZnSO4 and STX on SH-SY5Y cells.

*Cells were plated in 96 well plates and exposed to $ZnSO_4$ (0-100mM, **A**) or STX (0-10µg/L, **B**) for 24h with resorufin absorbance read at 0h (white bars), 12h (grey bars) and 24h (striped bars). Data is expressed as mean absorbance ± SD (n=3) and was subjected to two-way ANOVA with Tukey's multiple comparisons test. *p<0.05 compared to all $ZnSO_4$ concentrations at same time point (**A**) and medium only control at same time point (**B**).*

3.5 Discussion

The aim of the present study was to repeat and extend a previous study [18] to use resazurin to establish a continuous real time non-destructive cytotoxicity assay. The previous study used OVCAR-3 cells while the present study extended this work and included SH-SY5Y cells, and additionally tested this assay with known cytotoxic and non-cytotoxic substances. A wider range of OVCAR-3 cell densities, exposure times and resazurin concentrations were examined which provided a linear relationship between cell density and absorbance than in a previous report [18]. Having a wider range of exposure times make the assay more flexible and customizable.

For cells exposed to 175μM and 300μM resazurin there were linear increases in resorufin absorbance 0.06-0.5 x10^4 cells/well in addition to lower variation and significant increases in resorufin absorbance for all seeding densities (Fig 3.1B-C), suggesting these would be optimal conditions for a resazurin based assay in this cell line. Although there were linear ranges for OVCAR-3 cells exposed to 50μM resazruin there was large variation and significant increases in resorufin absorbance were only seen after 48h for the highest densities suggesting these conditions were not optimal (Fig 3.1A).

Again, although there were linear ranges at the highest resazurin concentration for the OVCAR-3 cells, the densities were narrowed, additionally using a lower concentration of resazurin makes for a more cost-effective assay and avoids potential cytotoxic effects which is evident by no production of resorufin at the lower densities and reduced production at higher densities and longer exposure periods. Whilst the rate at which

resorufin was produced was reduced at the highest concentration (Fig 3.1D) similar maximum ODs were still reached suggesting either resazurin toxicity to a percentage of the cells leaving a smaller number of cells to reach this maximum, which is supported by the greater production of resorufin at lower densities for the lower resazurin concentrations, or that the increased resazurin concentration slowed the cells ability to produce resorufin.

To test the use of a resazurin based assay to successfully identify cytotoxic agents the assay was compared to the well-known MTT assay (Fig 3.2). Comparable results were obtained with the resazurin based assay successfully identifying H_2O_2 as cytotoxic and with greater sensitivity. Further, resazurin toxicity was not observed with cell numbers increasing from 4 to 48h. Together this supports this use of a resazurin based assay in OVCAR-3 cells

Expanding to a second cell line, the SH-SY5Y cell line was investigated. Firstly the proliferation and doubling time of SH-SY5Y cells were monitored at increasing seeding densities using a trypan blue exclusion assay to measure cell numbers (Fig 3.3 and Table 3.1). Seeding densities used here were equivalent to $0.0625\text{-}0.25\text{x}10^4$ cells/ml in the 96 well plate format when considering surface area which is 3 times smaller with one fifth of the medium volume used. We found that the doubling time of SH-SY5Y cells was very rapid, ranging from 13 to 20h for the first 72h of culture for the 2 higher densities, and it was also noticeable that the proliferation rate increased as cell density increased. These two densities were almost parallel for the 120h.

In comparison to other cell lines, a 'fast' JAr cell line derived from a choriocarcinoma of the placenta has a doubling time of 15h [21] and embryonic stem cells have equivalent doubling times to the SH-SY5Y cells [22]. The doubling time of OVCAR-3 cells has been determined to be 48h [23], considerably longer than the doubling time of 13-20h we found for moderate SH-SY5Y cell densities under non-limited culture conditions. Since the reduction of resazurin to resorufin is dependent on the metabolic and proliferation rate of the cell line, this would suggest that OVCAR-3 cells would require longer exposure times to reduce the resazurin compared to SH-SY5Y cells. Although not in a cell culture set up pH has been shown to effect the rate at which resazurin is reduced to resorufin [24]. This could affect the results of extended exposure periods where the pH of medium is likely to decrease as cells proliferate. Given that SH-SY5Y cells proliferate at a faster rate than OVCAR-3 cells changes in pH will be inconsistent.

The large 6-well cell culture format with trypan blue exclusion assay indicated that $2.5x10^4$ SH-SY5Y cells/well doubled in 26h, after 24h in culture (Fig 3.3). The small 96 well format with resazurin detection showed an increase in OD value between 24 and 40h, but not a doubling in OD value. The relationship between cell number and resorufin-associated OD value was not direct or linear, and these data acknowledge that decreased cell metabolism and associated resorufin production may have been caused by resazurin toxicity as well as media depletion related to increased cell densities. Following 120h cell densities dropped for the 2 highest densities (Fig 3.3). Given that $2.5x10^4$ cells/well did not continue to increase to the same maximum as $5x10_4$ cells/well at 96h it suggested that the decrease was not simply a results of

media depletion and limitations on space but perhaps a maximum number of division following initial seeding.

While a resazurin based assay has the advantage of being able to monitor viability continuously on the same cells over an extended period of time it is limited by culture conditions. Medium cannot be refreshed during the assay resulting in nutrient depletion and adherence surface is limited in the wells of culture plates. We have shown using both the Trypan Blue and crystal violet assays that a loss in viability is unavoidable after extended culture periods and hence it is important to optimize cell density and exposure periods to avoid this. This inevitable decrease in viability after extended exposure periods is likely the major cause of the loss of linearity in the resazurin standard curves over time.

Before optimizing the assay in the SH-SY5Y cell line, the toxicity of resazurin was investigated (Fig 3.4). While the mechanism of action is unknown, here 100μM resazurin was significantly cytotoxic ($p<0.05$) at 40h but viability was not effected at 16h. Decreased viability following exposure to Alamar Blue (10% v/v, equivalent to 44μM) has also been recorded in other cell lines [1]. Two leukemia cell lines, Jurkat and HL-60, were exposed to resazurin at varying concentrations (22-88μM) and resulted in a decrease in proliferation which was greater in HL-60 cells [16]. Levels of resorufin in Jurkat cells were higher than HL-60 cells suggesting that it is not resorufin which is toxic rather resazurin-mediated ROS production which was higher in HL-60 cells. We have shown that OVCAR-3 cells exposed to 700μM resazurin had much

lower absorbance in the first 6hrs compared to the lower concentrations, supporting this theory that it is resazurin which is toxic.

When comparing the morphology of each cell line in the above mentioned study after exposure to resazurin both cell lines showed signs of apoptosis-like death which can be a result of autophagy and again the HL-60 cells were more affected. It appears that the Jurkat cells are more resistant to the effects of the dye, therefore it is important to choose an appropriate cell line when designing an assay with resazurin.

When optimizing the resazurin assay in the SH-SY5Y cells we expanded to the exposure time to 64h (Fig 3.5). We anticipated that 64h culture without replenishing the culture medium would limit cell proliferation and resazurin reduction, and found that production of resorufin increased in a linear fashion when the initial cell density was ≤ 4 x 10^4 cells/well. From our results the optimal conditions for SH-SY5Y cells were; 0.06-2x10^4 cells/well for 16 to 26h with 100μM resazurin. Given the large increase in OD values from 1 to 16h, this data set confirms that 100μM does not cause significant cytotoxicity during 16h. Additionally it does not appear that resazurin is cytotoxic to all cells as OD values continued to increase following 40h (Fig 3.4B). There is still a need to determine a shorter exposure period, between 1 and 16h, which will give a cell-dependent linear increase in resorufin or for an exposure period between 16 and 40h which will not result in cytotoxicity.

Confirming the crystal violet assay results (Fig 3.3) it appears that 8x10^4 is the maximum seeding density for this cell line as the crystal violet OD values did not increase from the initial 16h reading nor did the production of

resorufin increase here. This confirms the need for lower seeding densities in order to ensure that sufficient increases in OD are seen over the exposure period.

Although there were decreases in viability due to both the dye itself and the extended culture periods the assay could be useful for high throughput preliminary screening of compounds. This would be especially useful if compounds are scarce and/or expensive, and to identify a bracket of time when cell death occurs for later accurate examination with another assay. A previous study which noted anti-proliferative effects of AB suggest that these effects should not deter one from using AB and that it is suitable for short exposure periods with serum free medium [25]. Interference by serum and other medium constituents has additionally been reported by others [26].

An optimized assay was then tested with SY-SY5Y cells with two substances; $ZnSO_4$, known to be cytotoxic, and STX which is not cytotoxic in culture (Fig 3.6). A seeding density of $2x10^4$ cells/well was chosen as it fell within the linear range and measurement times of 12 and 24h were chosen as it was expected that OD value increases would be discernable and they were before 40h when significant cytotoxicity was measured.

As predicted there was a significant concentration-dependent decrease in the reduction of resazurin to resorufin following exposure to $ZnSO_4$ for 12 and 24h. As mentioned STX is not directly cytotoxic to cells but it was unknown if the toxin would have an effect on cellular metabolism. The constant increase in the reduction of resazurin to resorufin in the first 12h suggested that it had no effect on cellular metabolism and the lack of increase from 12 to 24h

suggests a maximum reduction has been reached. Further investigation of STX's affects would be beneficial but for the scope of this article, this experiment demonstrates that the assay was successfully optimized in SY-SY5Y cells and successfully identified a known cytotoxic compound, $ZnSO_4$.

While a resazurin based assay has the advantage of having a straightforward high-throughput protocol, which can provide a wealth of data and is quick and cheap in comparison to other available assays it also has its drawbacks.

Aside from the significant decrease in viable SH-SY5Y cells we also identified a large variation in results. We have shown a large amount of variability between repeats in the SH-SY5Y cells with inter-assay %CV being in the range of 16.3-20.7%, higher than previously recorded using AB [12] and higher than other calibrated assays such as the MTT assay [21].

In conclusion the assay optimized here has some drawbacks as do many others but given proper optimization it can be of benefit. Given the simplicity of the assay it is easily customizable to suit optimal conditions of each new cell line used. In addition to this assay it would be advantageous to use a variety of viability assays with different detection mechanisms in order to better understand the effects of a test compound on cells, which has previously been suggested [27].

To our knowledge this is the first report of a non-destructive cell metabolism and viability assay using resazurin that allows the same cell cultures to be assessed at multiple consecutive time points using SH-SY5Y cells. The assay format described herein is novel, inexpensive, technically simple and

requires only a plate spectrophotometer for data acquisition. Additionally, it is applicable to a wide variety of drug development scenarios.

3.6 Acknowledgments

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3.7 References

1. Squatrito, R.C., Connor, J.P., and Buller, R.E., *Comparison of a novel redox dye cell growth assay to the ATP bioluminescence assay.* Gynecol Oncol, 1995. **58**(1): p. 101-5.

2. Rampersad, S.N., *Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays.* Sensors (Basel), 2012. **12**(9): p. 12347-60.

3. Gillies, R.J., Didier, N., and Denton, M., *Determination of cell number in monolayer cultures.* Analytical Biochemistry, 1986. **159**(1): p. 109-113.

4. Yang, Y., et al., *Counterion-dye staining method for DNA in agarose gels using crystal violet and methyl orange.* Electrophoresis, 2001. **22**(5): p. 855-9.

5. Dutt, M.K., *Staining of depolymerised DNA in mammalian tissues with methyl violet 6B and crystal violet.* Folia Histochem Cytochem (Krakow), 1980. **18**(1): p. 79-83.

6. Kueng, W., Silber, E., and Eppenberger, U., *Quantification of cells cultured on 96-well plates.* Anal Biochem, 1989. **182**(1): p. 16-9.

7. Rothman, S.W., *Technique for measuring 50% end points in cytotoxicity assays for Clostridium difficile toxins.* J Clin Pathol, 1986. **39**(6): p. 672-6.

8. Scragg, M.A. and Ferreira, L.R., *Evaluation of different staining procedures for the quantification of fibroblasts cultured in 96-well plates.* Anal Biochem, 1991. **198**(1): p. 80-5.

9. Lison, D., et al., *Nominal and effective dosimetry of silica nanoparticles in cytotoxicity assays.* Toxicol Sci, 2008. **104**(1): p. 155-62.

10. Berry, J.M., Huebner, E., and Butler, M., *The crystal violet nuclei staining technique leads to anomalous results in monitoring mammalian cell cultures.* Cytotechnology, 1996. **21**(1): p. 73-80.

11. Kitagaki, M., et al., *Sirc-cvs cytotoxicity test: an alternative for predicting rodent acute systemic toxicity.* J Toxicol Sci, 2006. **31**(4): p. 371-9.

12. Al-Nasiry, S., et al., *The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells.* Human Reproduction, 2007. **22**(5): p. 1304-1309.

13. O'Brien, J., et al., *Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity.* Eur J Biochem, 2000. **267**(17): p. 5421-6.

14. Gonzalez, R.J. and Tarloff, J.B., *Evaluation of hepatic subcellular fractions for Alamar blue and MTT reductase activity.* Toxicology in Vitro, 2001. **15**(3): p. 257-259.

15. Jonsson, K.B., et al., *A New fluorometric assay for determination of osteoblastic proliferation: Effects of glucocorticoids and insulin-like growth factor-I.* Calcified Tissue International, 1997. **60**(1): p. 30-36.

16. Erikstein, B.S., et al., *Cellular stress induced by resazurin leads to autophagy and cell death via production of reactive oxygen species and mitochondrial impairment.* Journal of Cellular Biochemistry, 2010. **111**(3): p. 574-584.

17. Czekanska, E.M., *Assessment of cell proliferation with resazurin-based fluorescent dye.* Methods Mol Biol, 2011. **740**: p. 27-32.

18. Nakayama, G.R., et al., *Assessment of the Alamar Blue assay for cellular growth and viability in vitro.* Journal of Immunological Methods, 1997. **204**(2): p. 205-208.

19. Franklin, R.B. and Costello, L.C., *The Important Role of the Apoptotic Effects of Zinc in the Development of Cancers.* Journal of cellular biochemistry, 2009. **106**(5): p. 750-757.

20. O'Neill, K., Musgrave, I.F., and Humpage, A., *Extended Low-Dose Exposure to Saxitoxin Inhibits Neurite Outgrowth in Model Neuronal Cells.* Basic & Clinical Pharmacology & Toxicology, 2016: p. n/a-n/a.

21. Edwards, V., Benkendorff, K., and Young, F., *An in vitro high-throughput assay for screening reproductive and toxic effects of anticancer compounds.* Biotechnol Appl Biochem, 2014. **61**(5): p. 582-92.

22. Rathjen, J. and Rathjen, P.D., *Mouse ES cells: experimental exploitation of pluripotent differentiation potential.* Current Opinion in Genetics & Development, 2001. **11**(5): p. 587-594.

23. Hamilton, T.C., et al., *Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors.* Cancer Res, 1983. **43**(11): p. 5379-89.

24. Toumadje, A., et al., *Pluripotent differentiation in vitro of murine ES-D3 embryonic stem cells.* In Vitro Cell Dev Biol Anim, 2003. **39**(10): p. 449-53.

25. Desaulniers, D., et al., *Optimization of an MCF7-E3 Cell Proliferation Assay and Effects of Environmental Pollutants and Industrial Chemicals.* Toxicology in Vitro, 1998. **12**(4): p. 409-422.

26. Goegan, P., Johnson, G., and Vincent, R., *Effects of serum protein and colloid on the alamarBlue assay in cell cultures.* Toxicology in Vitro, 1995. **9**(3): p. 257-266.

27. Fotakis, G. and Timbrell, J.A., *In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride.* Toxicology Letters, 2006. **160**(2): p. 171-177.

4. Low dose exposure to saxitoxin affects neuronal differentiation of D3 embryonic stem cells.

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Publication Details	O'Neill, K., Musgrave I F., Humpage A. and Young F., *Low dose exposure to saxitoxin effects neuronal differentiation of D3 embryonic stem cells.*

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Contribution to the Paper	Performed all experimental work, interpreted data, wrote manuscript and acted as corresponding author.
Overall percentage (%)	
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 26/5/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate to include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Please cut and paste additional co-author panels here as required

4.1 Abstract

The potent neurotoxin saxitoxin (STX) belongs to a group of structurally related analogues produced by both marine and freshwater phytoplankton. The toxins act by blocking voltage-gated sodium channels stopping the inflow of sodium ions and the generation of action potentials and acute exposure can lead to paralysis and death by respiratory depression. Guidelines for acute exposure from both sources exist, yet chronic low dose exposure which is a possibility has not been thoroughly investigated. Previously, we have described the adverse effects of low dose extended exposure to STX on modal neuronal cells. Moving to a model which more accurately models mammalian neuronal differentiation we aimed to investigate the effect of the drinking water guideline level (3μg/L STX) and a typical algal bloom concentration (10μg/L STX) on D3 embryonic stem cell differentiation. Following previously described methods cells were differentiated into a neural lineage using retinoic acid. Cells were assessed by examination of morphological development of neuronal features, expression of gene markers (*oct4, nestin, mixL1, ß$_{III}$-Tubulin and MAP2*) and positive identification of neuronal cells with immunohistochemical staining of ß$_{III}$-Tubulin. Morphology results show a concentration dependent decrease in neuronal index scores following exposure to STX, increased expression of *nestin* and *MAP2*, whilst expression of *ß$_{III}$-Tubulin* was delayed. These results suggest that STX disrupted proper neuronal differentiation and that this pattern of exposure may be of concern to public health.

4.2 Introduction

Paralytic shellfish toxins (PSTs) are a group of structurally related naturally occurring toxins produced by both marine and freshwater phytoplankton, and include saxitoxin (STX), the gonyuatoxins (GTX) and the C-toxins, which all share a 3,4,6-trialkyl tetrahydropurine skeleton [1]. The PSTs act by reversibly binding to axonal voltage-gated sodium channels (VGSCs), blocking the inflow of sodium ions and subsequently the generation of action potentials so that neurons lose their ability to transmit electrical impulses via action potential [2, 3]. Additionally the PSTs act on calcium and potassium channels [4-6] and are capable of crossing the blood brain barrier (BBB) so that both peripheral and central neurons can be affected [7]. The result can be numbness, paralysis and even death by respiratory depression [1].

Exposure to marine PSTs occurs following the consumption of shellfish which have fed on phytoplankton which produce the PSTs and historically this pattern of exposure has been the focus of public health concerns, given the potentially fatal outcome. Monitoring and prevention programs are set up globally, with a maximum allowable PST concentration of 80μg STX equivalents/100g of shellfish tissue, to prevent poisonings from this source [8, 9]. However it is possible that low dose extended exposure to the PSTs is occurring in communities which rely heavily on a seafood diet, and in which more than the expected daily average is consumed for considerable periods of time, an issue which has been recognised by the European Food Safety Authority (EFSA) [10]. In these circumstances there may be no obvious symptoms of PST poisoning, but there may be more subtle sub-clinical adverse effects.

The PSTs are also produced by freshwater cyanobacteria globally [11-18] and can be found in water sources from which drinking water is sourced or used recreationally. Effective water treatments ensure that concentrations of the PSTs stay below an acute drinking water guideline of 3μg/L STX [19-21] which has been implemented in Australia, New Zealand and Brazil [22, 23] and to date there have been no acute poisonings or fatalities from this source of exposure [24]. Again it is possible that low dose extended exposure may be occurring from this source as well because spot checks of final drinking water have indicated that low concentrations of STX (0.5μg/L) can survive conventional treatment [19]. The toxins can persist in water for several months and low toxicity analogues can undergo transformation to more toxic structures, so that concentrations of individual analogues continuously vary [25]. The toxicity of a mixture of these toxins is largely defined by the more toxic analogues, even when present at lower concentrations than the less toxic analogues [26]. It is likely that the climate changes associated with global warming will favour the growth of the source cyanobacteria, possibly leading to subsequent increases in toxin production [27-29], so that their presence is likely to be an ongoing problem in the future.

Although low dose extended exposure to the PSTs is likely there has been little research into this pattern of exposure, despite the fact that neuronal electrical activity which is disrupted by the PSTs plays a critical role in neurodevelopment [30]. For example the structurally unrelated but similarly acting toxin tetrodotoxin (TTX) has been shown to disrupt regular neurite outgrowth [30, 31] and we have previously shown using model neuronal cells that concentrations of STX below the drinking water guideline can cause

significant adverse effects on cellular neuronal morphology [32]. In both PC-12 and SH-SY5Y cells we saw a concentration-dependent increase in the number of cellular projections per cell but a decrease in the percentage of the projections which were classified as axonal-like based on length.

Exposure to neurotoxins during the critical period of neurodevelopment has been shown to disrupt normal embryonic development and have long term effects on an individual [33] and we hypothesise that STX too may cause these effects.

D3 ES cells have previously been used to study the effects of developmental neurotoxins [34-36] and have been shown to express voltage gated Na^+ and Ca^{2+} channels [37, 38]. It is known that transmembrane ion distribution is vital for cellular homeostasis and induced electrical activity has been shown to increase neuronal differentiation with Ca^{2+} playing a role [39].

In vitro, D3 ES cells are undifferentiated pluripotent cells which represent ICM cells of an *in vivo* blastocyst approximately 3 to 6.5 d.p.c., around the time of implantation [34]. Murine ES cells grown in suspension culture in the absence of leukaemia inhibitory factor (LIF) aggregate to form embryoid bodies (EBs) which represent a post-implantation embryo [40] and their *in vitro* differentiation is an accepted model of *in vivo* embryogenesis [41].

Therefore we report here an investigation of the effect of STX at the drinking water guideline level (3μg/L) and at an algal bloom level (10μg/L) [42], 10 and 30nM respectively, on the differentiation of D3 murine embryonic stem (ES) cells down a ectodermal (neural) pathway. D3 cells will be directed towards neural differentiation using retinoic acid(RA). We hypothesise that

exposure to STX will alter sodium ion homeostasis with a consequent decrease in neuronal differentiation.

4.3 Materials and Methods

All chemicals and reagents were from Sigma Aldrich unless otherwise stated.

4.3.1 Cell culture

Undifferentiated D3 ES cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Australia) supplemented with 2mM L-glutamine, 1000U/ml penicillin and streptomycin, 1000U/ml LIF (Merck Millipore, Australia), 0.1mM β-mercaptoethanol and 20% foetal bovine serum (FBS). Cells were grown at 37°C with 5%CO_2 and passaged every second day. EBs were grown in DMEM/F12 (1:1) (Gibco, Australia) supplemented with 2mM L-glutamine, 1000U/ml penicillin and streptomycin, 0.1mM β-mercaptoethanol and 20% FBS. EB medium was replenished every second day unless otherwise stated.

4.3.2 Preparation of Retinoic Acid

RA was reconstituted in 100% dimethyl sulfoxide (DMSO) then further diluted in EB medium giving 0.01M RA in 10% DMSO and stored in the dark at -20°C until required. For experiments RA was further diluted in EB medium so that cells were exposed to a maximum of 0.001% DMSO.

4.3.3 Preparation of Saxitoxin

CRM-STX-f (National Research Council, Canada), a certified calibration solution, was diluted in EB medium to 30μg/L (10mM) and 100μg/L (30nM) and stored in the dark at 4^{o}C.

4.3.4 Induction and expansion of EB populations

On EB day 0 (EB0) 1x10^{6} D3 ES cells were seeded into each of 6 non-adherent 100mm petri dishes in 10ml EB medium. The remaining cells, designated EB0, had RNA extracted. Following 48h incubation ES cells, no longer suppressed by LIF, had aggregated into EBs which were distributed into 12 dishes in 10ml EB medium. Following 24h incubation 1 dish had RNA extracted, designated EB3, and the remaining 11 dishes continued incubation.

4.3.5 The effect of STX on ES cell neural differentiation

On EB day 4, EBs were viewed under the microscope and EBs from the 8 best dishes were distributed into 16 dishes and allocated to 1 of 4 treatment groups, giving 4 replicate dishes per treatment group. Treatments were; 1) Non-neuronal control in EB medium, 2) Neuronal control receiving 1μM RA 3) low dose STX receiving 3μg/L STX and 1μM RA and 4) high dose STX receiving 10μg/L STX and 1μM RA. EBs from one remaining dish were photographed, designated pre-treatment, and the remainder discarded.

On EB day 6 single EBs from one dish per treatment group were added to each well of four 24 well plates previously coated with 0.1% gelatine and containing 1ml EB medium with corresponding treatments (One 24 well plate per treatment group). EBs from one dish per treatment group had RNA extracted, designated EB6, and two dishes per treatment group remained in culture.

On EB day 8 EBs from one dish per treatment group were photographed. The remaining dish per treatment group had medium replaced with EB medium without any treatments in accordance with the 4-/4+ principle [37] and cultured for 24h. These final dishes had RNA extracted on EB day 9 which was designated EB9.

Media in 24 well plates were replaced with EB medium without any treatments on EB8 and were replenished every 2 days thereafter, until day 14 when media were replaced with serum free EB medium and incubated for a further 2 days.

The experiment was repeated on three separate occasions (n=3) for the first three treatment groups and once (n=1) for the fourth treatment group (STX 10μg/L).

Refer to Table 4.1 for clarification.

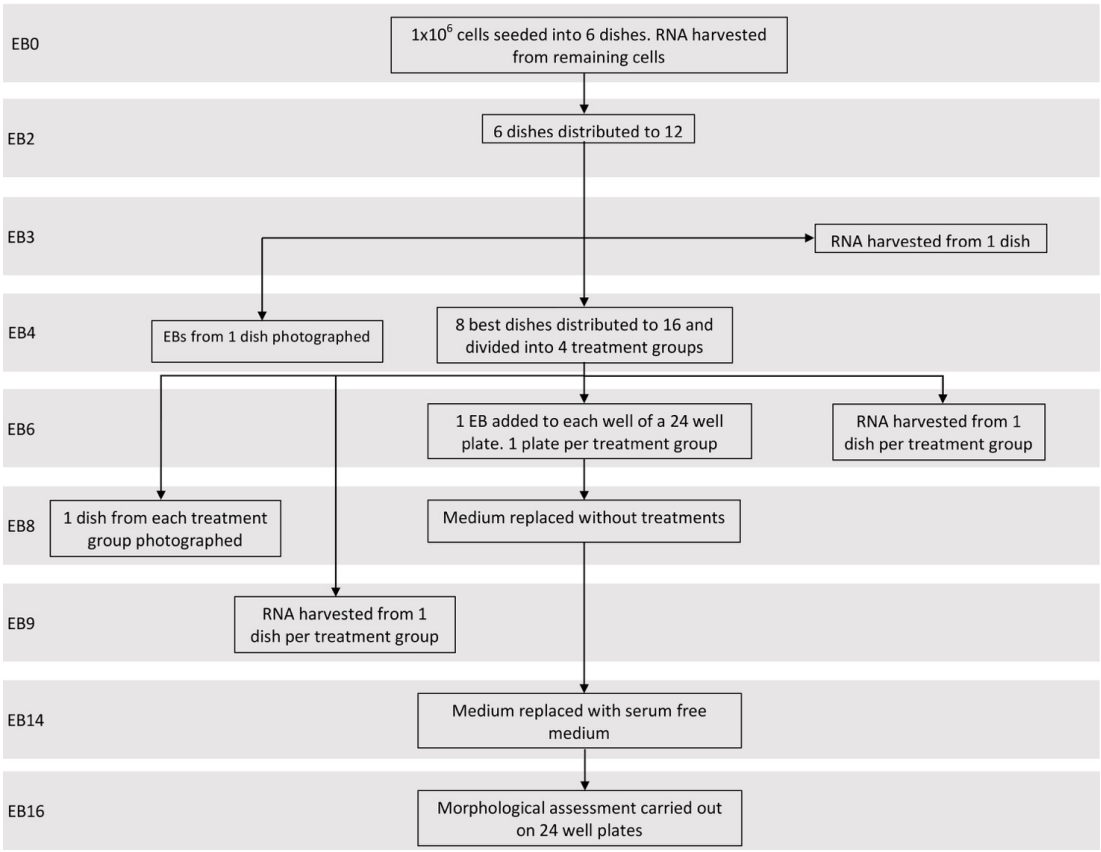


Table 4.1 Workflow of iv. Induction and expansion of EB populations and v. The effect of STX on ES cell neural differentiation

4.3.6 Morphological assessment

On EB day 16 cells in the 24 well plates were assessed for neural-like morphology. A Nikon IMT2 Inverted microscope with Dino-Eye AM7023 camera was used to capture images of cells through the x10 lens. Each well was assigned a confluence score of 1-3 where 1= low confluence, 2= intermediate confluence and 3= high confluence. Degree of neuronal development was assigned as score of 1-5 where 1= slightly elongated cell types, 2= elongated cells types resembling axons, 3= typical neural-like morphology of cells with cell body, axons and dendrites, 4= morphology suggestive of neural tubes and 5= neural network-type structures [34]. These

two scores were multiplied to give a neuronal index score for each well, which were averaged to give a mean neuronal index score per treatment group.

EB sizes were measured on EB days 4 and 8 using the same microscope and camera as described above. Diameters of EBs were measured using ImageJ (National Institutes of Health, USA) and measurements were averaged to give a mean diameter per treatment group. EB size was measured on one occasion (n=1)

4.3.7 RNA extraction and quantification

RNA was extracted using a Maxwell 16 LEV simplyRNA Cells Kit (Promega, Australia) according to the manufacturer's instructions on an AS2000 Maxwell 16 Instrument (Promega, Australia). Briefly cells and EBs were harvested on EB0, 3, 6 and 9, 200μl homogenisation solution was added to each and the cells were vortexed until pellets were dispersed, 200μl lysis buffer was added to each sample and vortexed for 15 seconds before being placed on ice. 400μl of each sample was loaded into a cartridge provided with the kit followed by addition of 50μl nuclease free water and 5μl DNase1 solution. Cartridges were placed in the Maxwell 16 instrument for automated RNA extraction. Following the extraction, the RNA was stored at -70^{0}C in Nuclease-free water.

To quantify RNA, 2μl of each sample was added to a Take-3 micro-volume plate. A Synergy MX Spectrophotometer (Millennium Science, Bio Tek) and Take-3 software was used to determine the concentration (ng/μl) of RNA.

4.3.8 Production of cDNA

Total EB RNA was reverse transcribed into cDNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to manufacturer's instructions. Briefly, 10μl reverse transcriptase buffer, 1μl reverse transcriptase enzyme mix, 2μg RNA and nuclease free water to make up 20μl were added to each well of a PCR quality white 96 well plate. The 96 well plate was centrifuged briefly at 1000g and placed on ice. Reverse transcription was carried out using a FX96 Touch Real-Time PCR Detection System (Biorad) at 37°C for 60min then contents of each well were transferred to sterile eppendorf tubes, quantified as described above, adjusted to a working concentration of 25ng/μl and stored at -20^{0}C.

4.3.9 Real time semi quantitative PCR

Expression of *Oct4*, *MixL1*, *Nestin*, *ß$_{III}$-tublin* and *MAP2* were measured relative to *Actb* using TaqMan Real-Time PCR gene expression assay kits (Predesigned validated primer and probe sets, Thermo Scientific, Australia) according to the manufacturer's instructions. Briefly, reactions were in 20μl in PCR quality white 96 well plates with 1μl FAM-labelled TaqMan gene of interest (GOI), 1μl VIC-labelled TaqMan *Actb*, 10μl iTaq universal probes supreme (Biorad), 4μl RNase-free water and 4μl cDNA (100ng). The 96 well plate was centrifuged briefly at 1000g and placed on ice until use. PCR reactions were performed using a CFX96 Touch Real-Time PCR Detection System (Biorad). The reaction conditions were 98°C for 3m then 49 cycles of

95^0C for 55s and 60°C for 30s. The quantification cycle (Cq) was determined as the mean of 3 technical replicates by using CFX Manager software. ΔCq expression values (GOI expression relative to *Actb*) were calculated using equation 4.1 below:

$$\Delta Cq \text{ expression} = 2 \times -(C_{q\ GOI} - C_{q\ Actb})$$

Equation 4.1

4.3.10 Statistical analysis

The mean neuronal index scores and mean ΔCq of gene expression ± SEM of 3 repeated experiments were analysed using GraphPad Prism 6 with one and two-way ANOVA and Sidak's and Tukey's multiple comparisons test respectively. Significance was assigned at $p<0.05$.

4.4 Results

4.4.1 Neuron-like morphology

The lowest neuronal index scores were for the non-neuronal control cells (2.1±0.05, Figure 4.1) and the highest were for the neuronal control cells (5.5 ±0.3, Figure 4.1). Non-neuronal control cells were circular with limited short projections (Figure 4.2A and B). In comparison the neuronal control cells were elongated in appearance with multiple long cellular projections (Figure 4.2C and D) and had significantly greater neuronal index scores compared to non-neuronal controls ($p<0.01$). These cellular projections were often branched and had made connections with neighbouring cells.

Exposure to 3μg/L STX reduced the neuronal score by 24% (Figure 4.1). The presence of STX during differentiation gave rise to circular cells which had shorter projections (Figure 4.2E) as well as cells with typical neuronal appearance (Figure 4.2F). However, these cells with typical neuronal appearance appeared to be isolated and had not made connections with neighbouring cells.

Exposure to 10μg/L STX reduced the neuronal score by 59% (Figure 4.1) to a score similar to the non-neuronal control. Similar to 3μg/L the higher concentration of STX resulted in cells which were circular (Figure 4.2G) and cells with typical neuronal appearance with long extensions but were not arranged in advanced networks (Figure 4.2H).

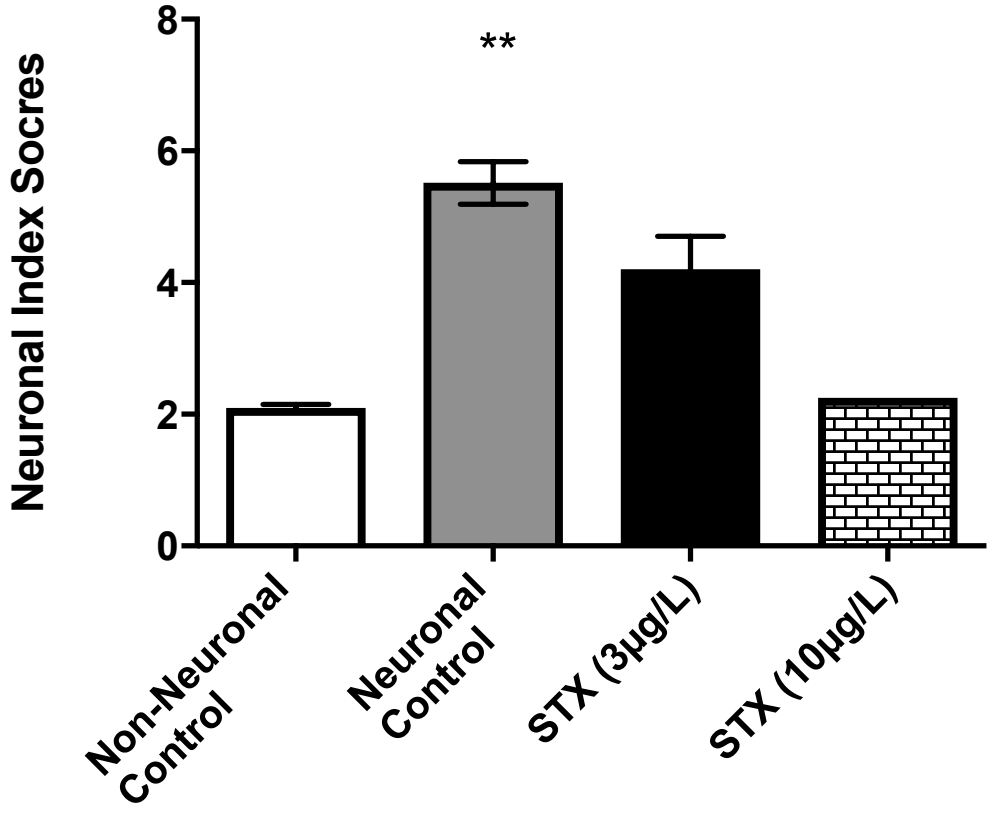
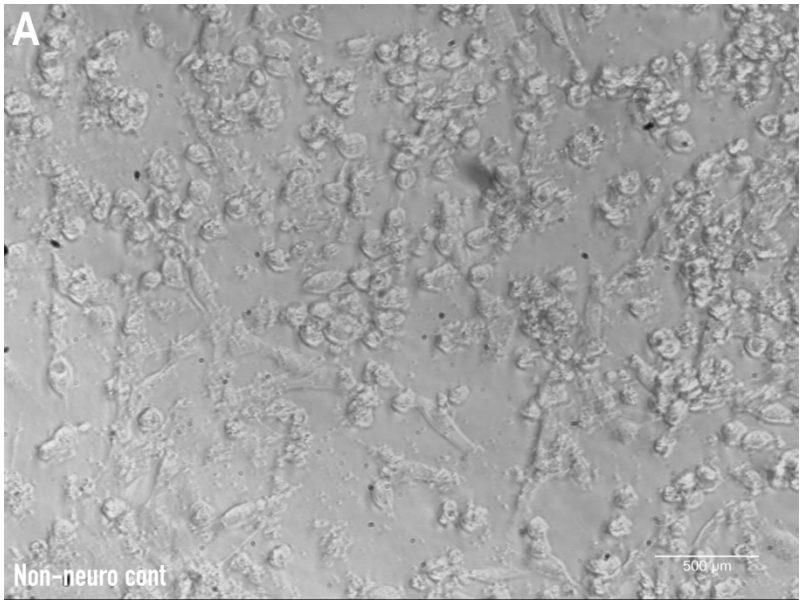
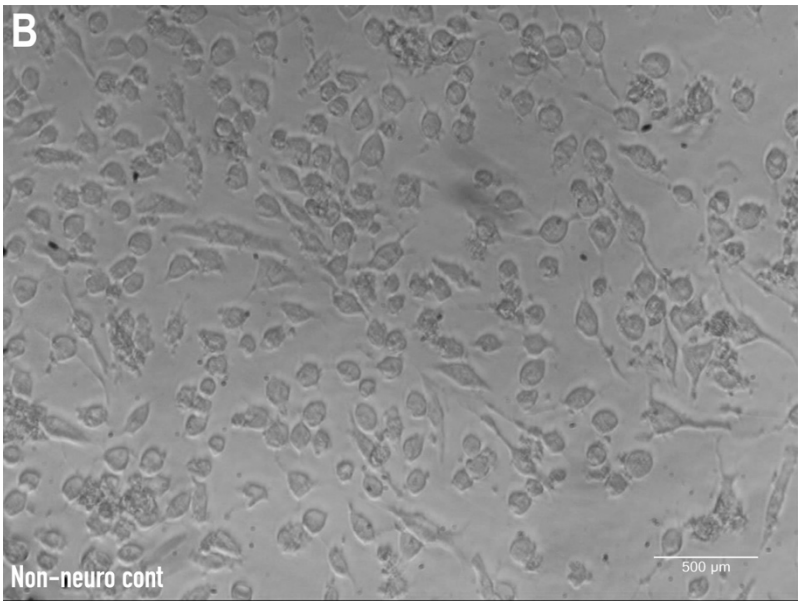
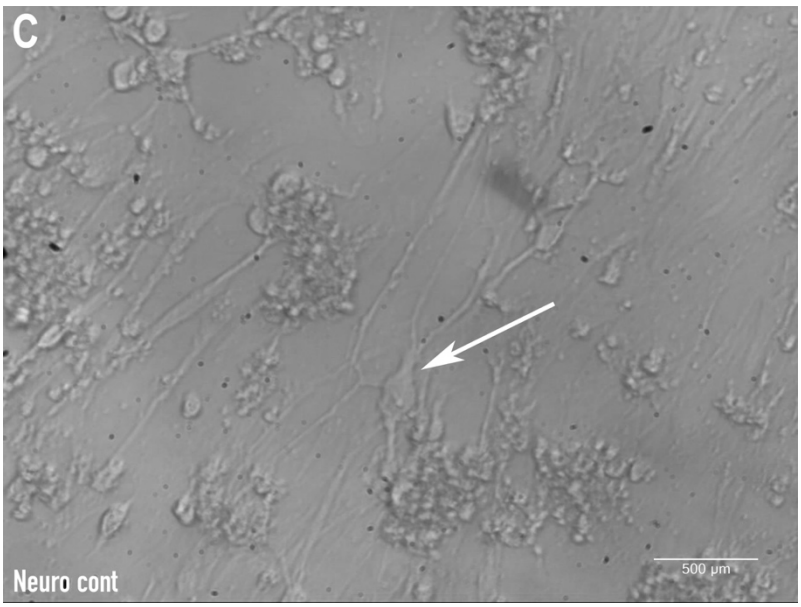


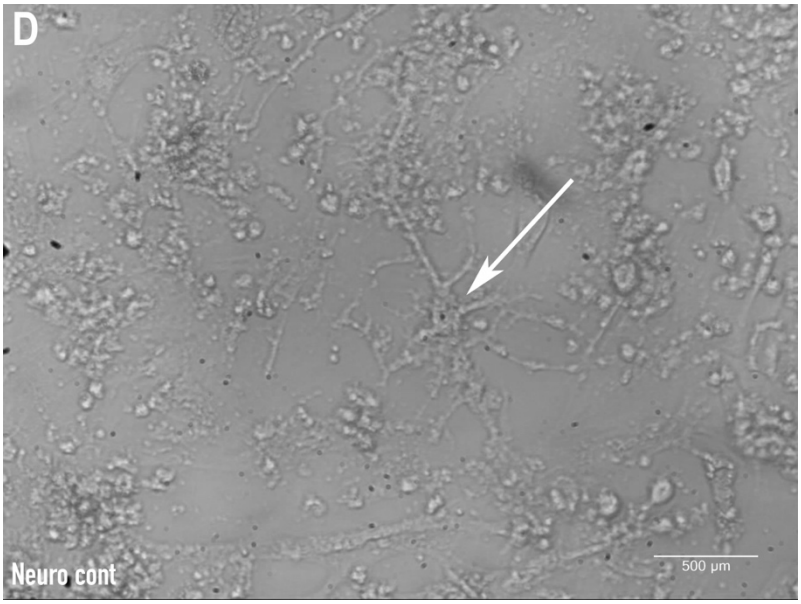
Figure 4.1 Effect of STX on morphology of embryonic stem cells during neuronal differentiation.

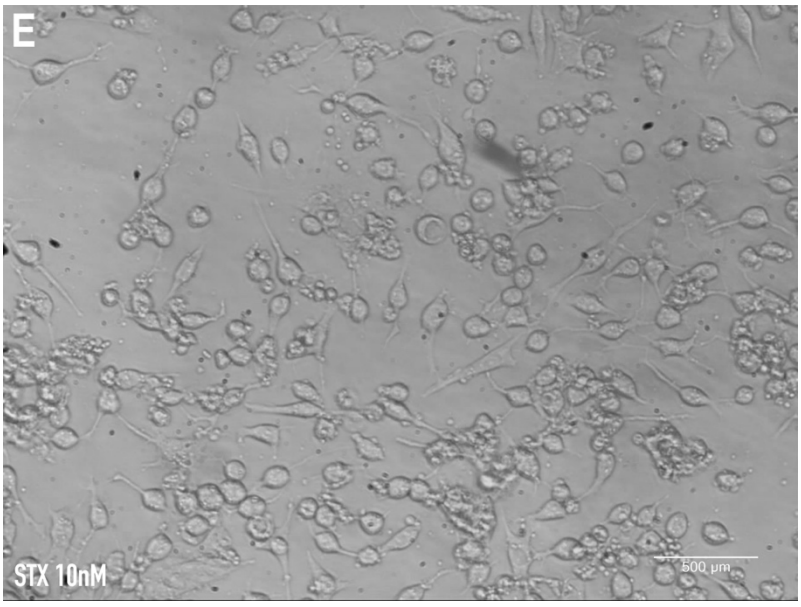
*Embryoid bodies (EBs) were grown on 0.1% gelatine in 24 well plates and exposed to culture medium alone, 1µM retinoic acid (RA), 1µM RA with 3µg/L STX or 1µM RA with 10µg/L STX from EB4 to EB8, then cultured for an additional 8 days before microscopic examination on EB16. Each well was assigned a confluence score of 1-3 where 1= low confluence, 2= intermediate confluence and 3= high confluence. Degree of neuronal development was assigned as score of 1-5 where 1= slightly elongated cell types, 2= elongated cells types resembling axons, 3= typical neural-like morphology of cells with cell body, axons and dendrites, 4= morphology suggestive of neural tubes and 5= neural network-type structures. Confluence and degree of development scores were multiplied together to produce a neuronal index score for each well. Data is expressed as mean index scores ± SEM, n=3. Morphological data for medium control, RA control and 1µM RA with 3µg/L STX were subjected to One-Way ANOVA with Sidak's multiple comparisons test comparing treatments to non-neuronal control, *p≤0.05, **p≤0.01.*

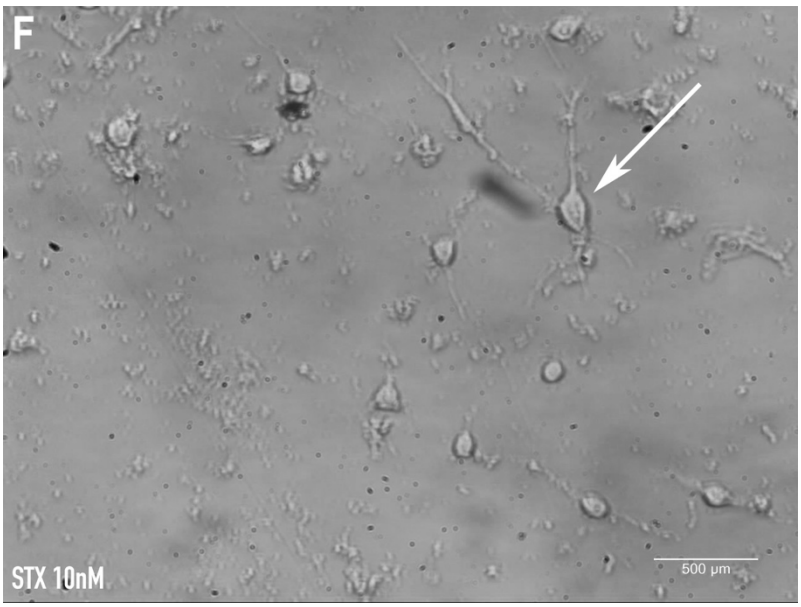

A
Non-neuro cont
500 µm


B
Non-neuro cont
500 µm


C
Neuro cont
500 µm


D
Neuro cont
500 µm


E
STX 10nM
500 µm


F
STX 10nM
500 µm

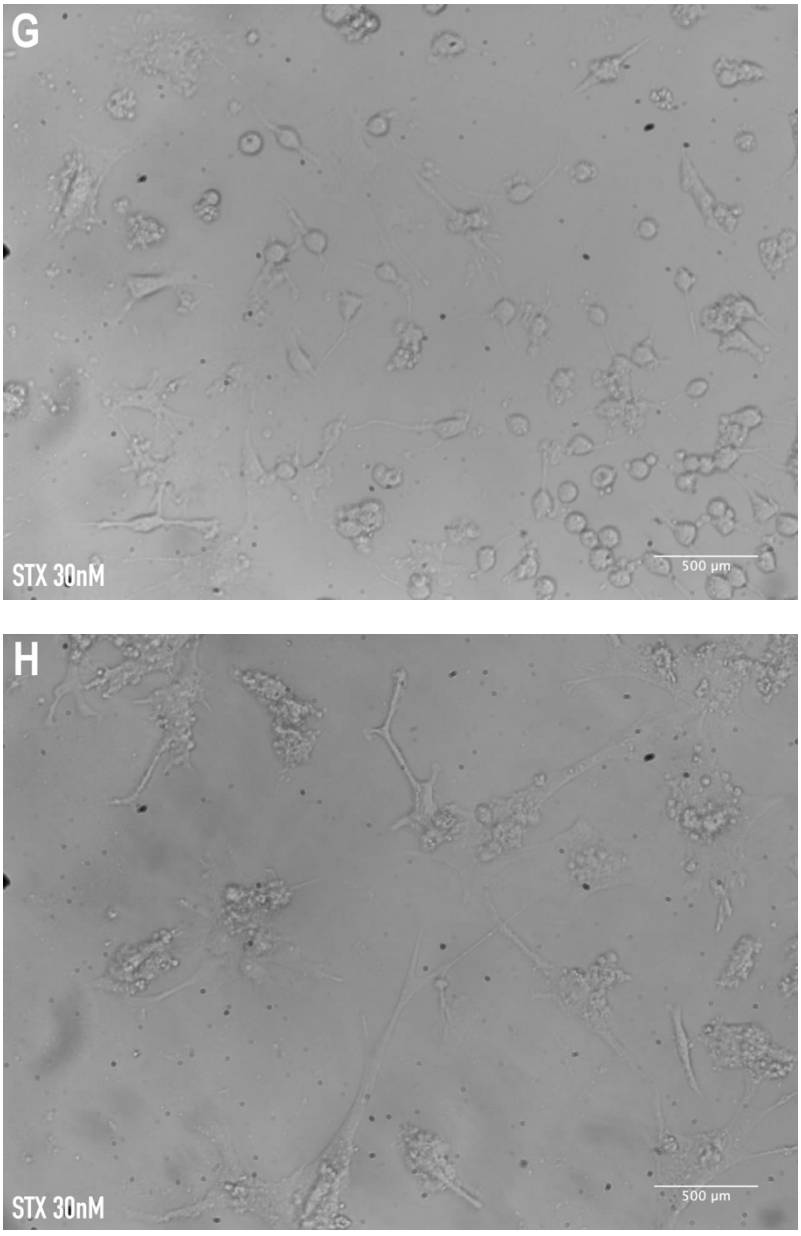


Figure 4.2 Effect of Saxitoxin (STX) on morphology of embryonic stem cells during neural differentiation.

Embryoid bodies (EBs) were exposed to culture medium alone (A and B), 1µM retinoic acid (RA) (C and D), 1µM RA with 3µg/L STX (E and F) or 1µM RA with 10µg/L STX (G and H) from EB4 to EB8, then cultured for an additional 8 days in 24 well plates coated with 0.1% gelatine before microscopic examination on EB16. Cells were photographed on a Nikon IMT2 inverted microscope at 10x. Arrows indicate cells with typical neuronal morphology. Bar = 500µm

4.4.2 EB growth

From EB4 to EB8 the size of EBs increased by 62% in neuronal controls (Table 4.2). Compared to day 8 neuronal controls exposure to 3μg/L STX and 10μg/L reduced expected growth of EBs by 44% and 35% respectively.

EB4	EB8		
Pre-treatment	Neuronal control	RA + 3μg/L STX	RA + 10μg/L STX
839±25μm	1362±87μm	761±43μm	881±90μm

Table 4.2 Effect of Saxitoxin (STX) on size of embryoid bodies (EB).

Diameter of EBs grown in EB media for 4 days followed by exposure to culture medium alone ("Pre-treatment"), 1μM retinoic acid (RA) (Neuronal control), 1μM RA plus 3μg/L STX or 1μM RA plus 10μg/L STX for a further 4 days. Data is expressed as mean ± SEM of technical replicates (n=1).

4.4.3 Relative gene expression

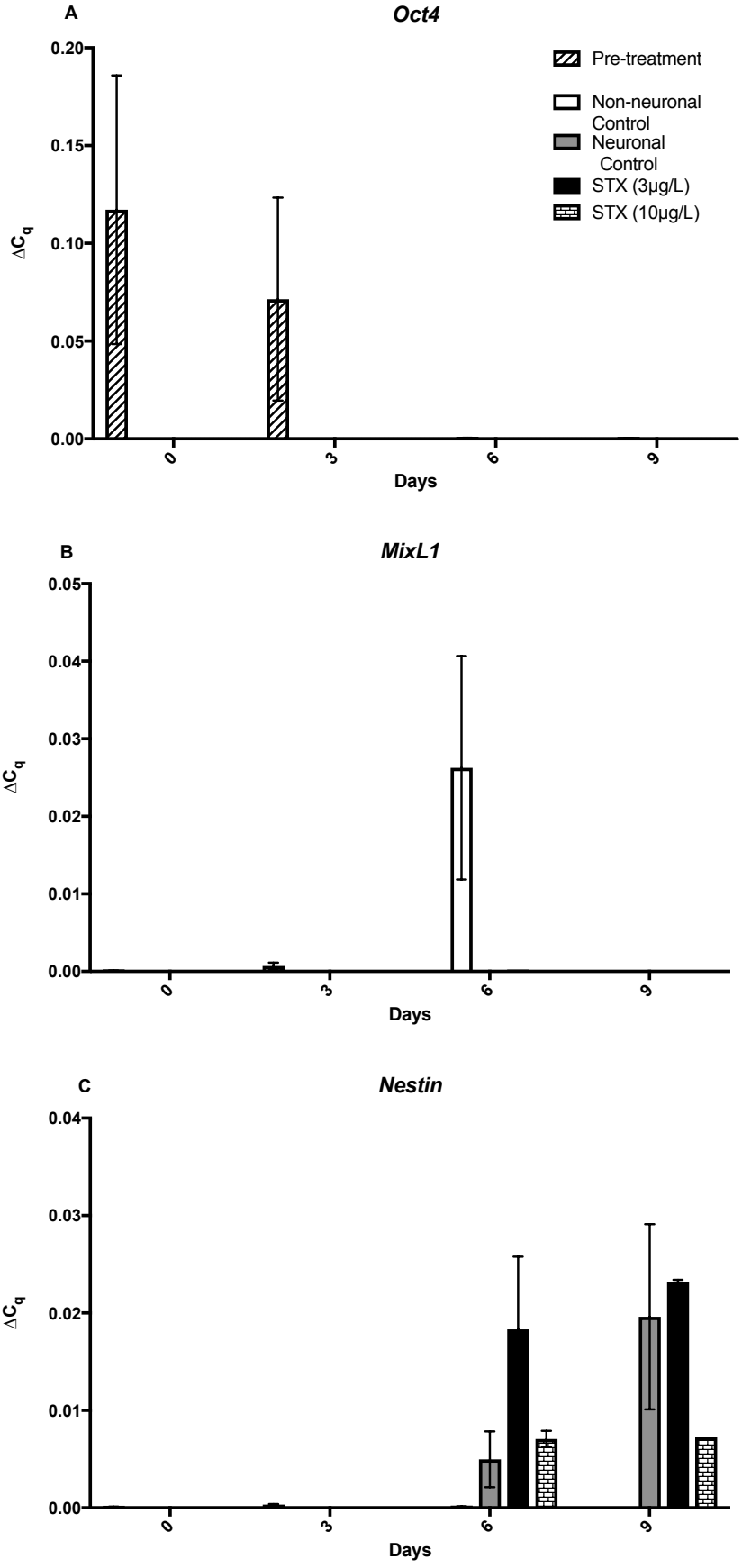
The undifferentiated cell marker *Oct4* had the highest relative expression on EB0 (Figure 4.3A). Expression decreased at each of the following days in the non-neuronal control and was not present on EB6 or EB9 for the 3 treatment groups directed towards neuronal differentiation with RA. The non-neuronal mesoderm marker MixL1 expression increased from EB0 to EB6 in non-neuronal controls, where it was significantly greater than all other treatments groups and times, before rapidly decreasing on EB9. There was negligible MixL1 expression by cells directed towards neuronal differentiation with RA (Figure 4.3B).

Nestin, expressed in neuronal precursor cells, was increasingly expressed on EB6 and EB9 following exposure to RA (Figure 4.3C). There was also an

increase in *nestin* on both EB6 and EB9 following exposure to 3*μ*g/L STX whereas exposure to 10*μ*g/L STX stalled *nestin* expression on EB6.

The neuronal marker *ß$_{III}$-tubulin* was expressed on pre-treatment days EB0 and EB3 and decreased on each following day in non-neuronal control cells (Figure 4.3D). Exposure to RA increased expression on each day and greatest expression was seen in neuronal control cells. Exposure to STX delayed expression of *ß$_{III}$-tubulin* to EB9.

Similar to *Nestin* and *ß$_{III}$-tubulin*, *MAP2*, the mature neuronal marker, was increasingly expressed on EB6 and EB9 following treatment with RA in neuronal control cells and cells exposed to 3*μ*g/L STX, with expression being significantly greater on EB9 in cells exposed to 3*μ*g/L STX (Figure 4.3E). Expression in cells exposed to 10*μ*g/L STX was similar to pre-treatment levels.


A
Oct4
Pre-treatment
Non-neuronal Control
Neuronal Control
STX (3µg/L)
STX (10µg/L)
ΔCq
0.20
0.15
0.10
0.05
0.00
0
3
6
9
Days
B
MixL1
ΔCq
0.05
0.04
0.03
0.02
0.01
0.00
0
3
6
9
Days
C
Nestin
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0.01
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0
3
6
9
Days

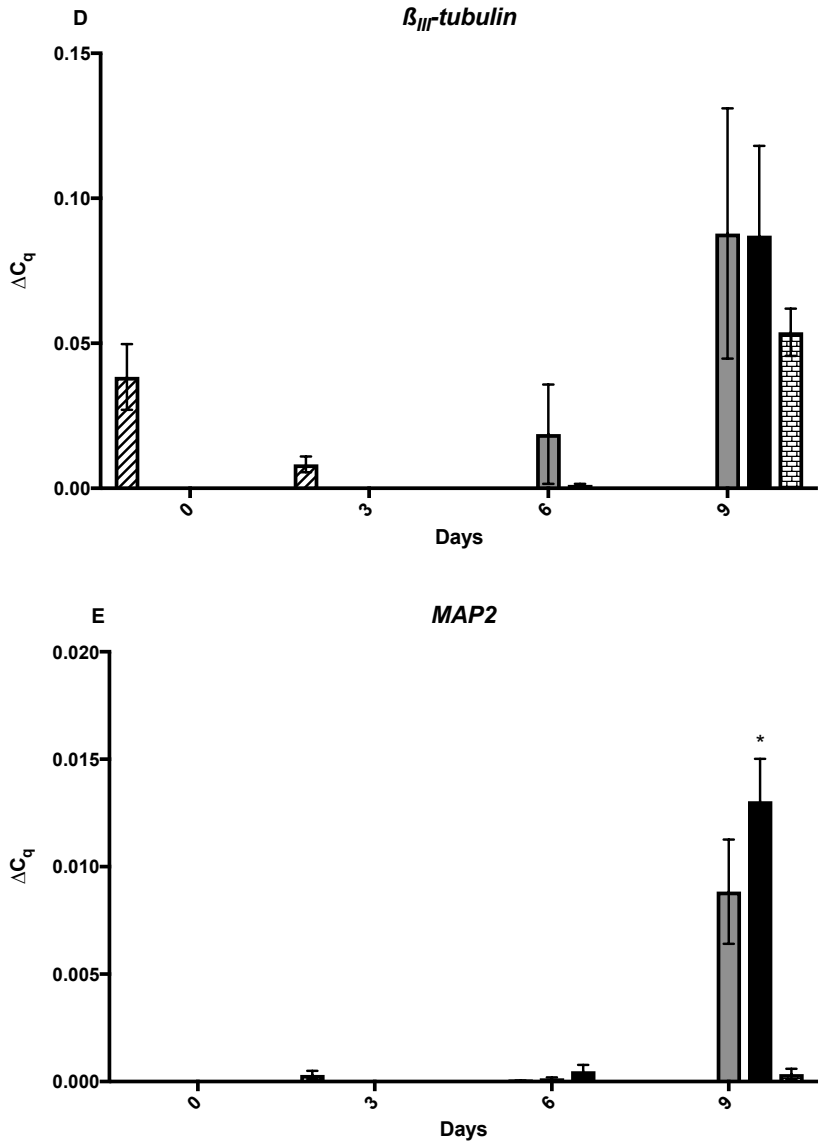

Figure 4.3 Figure 4.3 Effect of Saxitoxin (STX) on gene expression in embryonic stem cells during neural differentiation.

*Embryoid bodies (EBs) were exposed to culture medium alone (White bars), 1μM retinoic acid (RA) (Grey bars), 1μM RA with 3μg/L STX (Black bars) or 1μM RA 10μg/L STX (Brick bars) from EB4 to EB8. RNA was extracted from cells on EB0, 3, 6 and 9 after withdrawal of leukaemia inhibitory factor. 2μg of each sample was reverse transcribed and 100μg cDNA was analysed using TaqMan Real-Time PCR gene expression assay kits; Oct4 (**A**), MixL1 (**B**), Nestin (**C**), ß$_{III}$-tubulin (**D**) and MAP2 (**E**) with ß-actin as an internal control. Data is expressed as mean ΔCq ± SEM, n=3 for 1μM RA and 1μM RA with 3μg/L STX, n=2 for culture medium control and n=1 for 1μM RA with 10μg/L STX. ΔCq data were subjected to Two-Way ANOVA with Tukeys's multiple comparisons test where n=3. *p<0.05 compared to non-neuronal control for same day.*

4.5 Discussion

Extended exposure to low doses of the PSTs has not received much attention. We have investigated the effect of low dose (3μg/L) STX and an expected bloom concentration (10μg/L) on the differentiation of D3 ES cells into a neural lineage, to date the first investigation of its kind.

Firstly, STX reduced the growth of EBs. This failure to grow suggests that STX slowed cell division or caused cell death within the EBs. If STX caused cell death, then the EBs which were plated into the 24 well plates comprised cells which have survived the effects of STX and may have continued to grow from EB8 to EB16 in the same manner as the neuronal controls. However, the concentration dependent decrease in neuronal scores suggests that STX had a lasting effect on the cells which survived the initial exposure period.

Secondly, exposure to STX at the guideline level reduced neuronal index scores of cells directed towards neuronal differentiation and confirmed previous work [32] showing that STX had an adverse effect on the ability of cells to develop neuronal morphology. Previously the numbers and lengths of axonal like projections per cell were decreased by STX (0.25-3μg/L). This requirement for cellular sodium ion homeostasis for neuronal outgrowth has also been recorded by others [43].

STX affected both the degree of neuronal differentiation and the confluence of cells with neuronal-like appearance. There were instances when there were many cells with very limited neuronal morphology, remaining circular in appearance with short projections, as well as instances of isolated cells with

advanced neuronal morphology. This suggests that STX is not completely inhibiting the cells from developing advanced neuronal morphology, but disrupting the process. This change to shorter projections will have functional implications for the cells as shorter extensions such as filopodia and longer axonal extensions serve different functions [44].

Where cells have progressed to advanced neuronal morphology their isolation may be caused by STX-activity at VGSC with consequent impairment of intercellular communication. In a developing nervous system this formation of connections between neurons and their targets is critical. Cells with advanced neuronal morphology which are isolated and unconnected to surrounding cells equates to fewer axonal links with other neurons and target tissue.

While the cellular morphology in this study paralleled earlier studies with model neuronal cells, the changes to gene expression were more complex. Oct4, expressed almost exclusively by undifferentiated ES cells, maintains the pluripotency of cells [45] and its expression is down-regulated as cells differentiate [46]. Oct4 is seen in EBs in vitro from days 1-6 following the removal of LIF [47]. Oct4 expression was greatest on EB0. As cells aggregated to form EBs the expression of *Oct4* decreased demonstrating that cells were losing their pluripotency and differentiation had been initiated. This is comparable to previous studies showing a time dependent decrease in *Oct4*, with it being undetectable at day 6 [47].

MixL1, a homeobox gene, is expressed in the primitive streak during gastrulation by precursor cells for the mesodermal and endodermal lineages

[48], has restricted expression in EBs between days 3 and 4 [49] and can be used as a negative marker of neural differentiation. Expression of *MixL1* was seen on EB6 in non-neuronal control cells. Expression of *MixL1* has been seen between embryonic day 5.5 and 11.5 in whole mouse embryos [50] but has been shown to be expressed earlier, EB4, in culture [49].

Nestin, which codes for an intermediate filament protein [51], is expressed in neural precursor cells [52] increasingly from days 3-9 in cells directed towards neural differentiation with retinoic acid (RA) [53] and is an early neuronal marker. As neuronal precursor cells progress into neuronal cells they express the neuron specific cytoskeleton proteins *ß$_{III}$-Tubulin* and *MAP2* [36, 54-56], markers for immature and mature neural (ectoderm) cell differentiation respectively [57], with expression of both seen increasingly in EBs from day 5 [58].

There was comparable expression in the present study in neuronal control cells with the exception of ß$_{III}$-Tubulin, as described above.

For cells exposed to RA and 3*µ*g/L STX, the expression of *nestin* and *Map2* was greater than in cells exposed to RA alone, suggesting that STX enhanced neuronal differentiation whilst delaying *ß$_{III}$-Tubulin* expression. Given the role of *Nestin* and *MAP2* proteins in cellular structure and growth, changes to morphology are not surprising following changes to expression. Further, the nestin protein, an intermediate filament (IF) protein, has an important role in cellular structure during the cell cycle to reorganise filament networks, controlling dynamic changes in cell ultrastructure [51]. Additionally, this protein may be associated with trafficking within progenitor cells. The ß$_{III}$-

tubulin protein is specifically expressed in post-mitotic differentiating neurons [36], and together with α-tubulins forms heterodimers which make up microtubules (MTs)[54]. The $ß_{III}$-tubulin protein is upregulated in microtubules as axons and dendrites continue to grow and mature and mutation studies have shown $ß_{III}$-tubulin to have a role in the growth and/or guidance of axons in the brain and spinal cord [55]. *MAP2* codes for microtubule-associated protein 2 (MAP2) which binds to MTs, stabilising them and allowing MT bundling to take place.

However, the direction of the change in expression was unexpected given the reduced morphology scoring and our previous work where we also recorded an adverse effect of STX on neuronal-like morphology. Activity during early development plays a role in feedback loops regulating developmental activities [59], this increased expression of neuronal makers could be a result of STX's disruption of this electrical activity causing unchecked increases in expression forcing the cells into an uncoordinated differentiation altering their subsequent development.

In comparison to the lower dose of STX, exposure to 10*μ*g/L STX resulted in reduced expression of neuronal markers *nestin* and *$ß_{III}$-Tubulin* and expression of *MAP2* was almost completely switched off suggesting that the higher concentration of STX had an inhibitory effect on neuronal differentiation consistent with the morphology scoring.

It is worth noting that given that gene expression was measured on EB9 and earlier and morphology was graded on EB16, expression may have decreased in the STX treated and at EB16 gene expression may reflect the

decreased neuronal scores. Extending measurements of gene expression for the length of the protocol would be advantageous in the future. Additionally, although neuronal gene expression was seen on EB9 that doesn't necessarily translate to protein production. There may be a longer lag time before proteins are assembled and then have actions within the cell and a longer protocol may be needed to see the carry on effect in morphology.

Whilst the best-known action of STX is blockade of the VGSC, it has also been shown to block voltage-gated calcium channels (VGCC) [4, 6]. Spontaneous calcium activity during early developmental stages may play a role in feedback loops which regulate intrinsic excitability and subsequently the expression of excitatory and inhibitory neurotransmitters, axon pathfinding and dendritic outgrowth [59]. It is therefore possible that STX's activity at VGSC and VGCC is disrupting this regulation of excitability causing downstream effects within the cells that culminates in altered morphology.

The results presented here are of concern for public health as the low concentration used here is the drinking water guideline and is also below the current seafood safety guidelines. However further investigation is needed as any adverse effects seen in humans following exposure will depend on the toxicokinetics of STX. Previously it has been shown that a single intravenous injection of 10μg/kg STX to cats resulted in concentrations of 1.81ng/g and 2.5ng/g STX in the brain and medulla oblongata respectively [7].

It is currently unknown if the PSTs are capable of crossing the placental barrier to reach a developing foetus. However during the progression from a

single celled zygote to a hatched peri-implantation blastocyst, which takes place in approximately 6 days in humans, embryos are bathed in the luminal fluid of the oviduct and uterus and it is possible for toxins to reach this luminal fluid following ingestion [34].

In conclusion we have shown that STX at the Drinking water guideline concentration (3*μ*g/L) affected murine ES cell neuronal differentiation *in vitro*. Whilst further work is needed, this work raises question about exposure to STX and its analogues during neuronal development and highlights the lack of research into this pattern of exposure.

4.6 Acknowledgments

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4.7 References

1. Wiese, M., et al., *Neurotoxic alkaloids: saxitoxin and its analogs.* Mar Drugs, 2010. **8**(7): p. 2185-211.

2. Terlau, H., et al., *Mapping the site of block by tetrodotoxin and saxitoxin of sodium channel II.* FEBS Letters, 1991. **293**(1,2): p. 93-96.

3. Savio-Galimberti, E., Gollob, M.H., and Darbar, D., *Voltage-gated sodium channels: biophysics, pharmacology, and related channelopathies.* Front Pharmacol, 2012. **3**: p. 124.

4. Jones, S.W. and Marks, T.N., *Calcium currents in bullfrog sympathetic neurons. I. Activation kinetics and pharmacology.* J Gen Physiol, 1989. **94**(1): p. 151-67.

5. Wang, J., Salata, J.J., and Bennett, P.B., *Saxitoxin is a gating modifier of HERG K+ channels.* J Gen Physiol, 2003. **121**(6): p. 583-98.

6. Su, Z., et al., *Saxitoxin blocks L-type ICa.* J Pharmacol Exp Ther, 2004. **308**(1): p. 324-9.

7. Andrinolo, D., Michea, L.F., and Lagos, N., *Toxic effects, pharmacokinetics and clearance of saxitoxin, a component of paralytic shellfish poison (PSP), in cats.* Toxicon, 1999. **37**(3): p. 447-464.

8. Van Dolah, F.M., *Marine algal toxins: origins, health effects, and their increased occurrence.* Environ Health Perspect, 2000. **108 Suppl 1**: p. 133-41.

9. Etheridge, S.M., *Paralytic shellfish poisoning: Seafood safety and human health perspectives.* Toxicon, 2010. **56**(2): p. 108-122.

10. EFSA, *Marine biotoxins in shellfish - summary on regulated marine biotoxins. Scientific Opinion of the Panel on Cantaminants in the Food Chain.* The EFSA Journal, 2009. **1306**: p. 1-23.

11. Ikawa, M., et al., *Comparison of the toxins of the blue-green alga Aphanizomenon flos-aquae with the Gonyaulax toxins.* Toxicon, 1982. **20**(4): p. 747-752.

12. Humpage, A.R., et al., *Paralytic shellfish poisons from Australian cyanobacterial blooms.* Aust. J. Mar. Freshwater Res., 1994. **45**(5): p. 761-771.

13. Onodera, H., et al., *New saxitoxin analogues from the freshwater filamentous cyanobacterium Lyngbya wollei.* Natural Toxins, 1997. **5**(4): p. 146-151.

14. Lagos, N., et al., *The first evidence of paralytic shellfish toxins in the freshwater cyanobacterium Cylindrospermopsis raciborskii, isolated from Brazil.* Toxicon, 1999. **37**(10): p. 1359-1373.

15. Pereira, P., et al., *Paralytic shellfish toxins in the freshwater cyanobacterium Aphanizomenon flos-aquae, isolated from Montargil reservoir, Portugal.* Toxicon, 2000. **38**(12): p. 1689-1702.

16. Pomati, F., et al., *The Freshwater Cyanobacterium Planktothrix Sp. FP1: Molecular Identification and Detection of Paralytic Shellfish Poisoning Toxins.* Journal of Phycology, 2000. **36**(3): p. 553-562.

17. Liu, Y., et al., *First report of aphantoxins in China—waterblooms of toxigenic Aphanizomenon flos-aquae in Lake Dianchi.* Ecotoxicology and Environmental Safety, 2006. **65**(1): p. 84-92.

18. Ballot, A., Fastner, J., and Wiedner, C., *Paralytic shellfish poisoning toxin-producing cyanobacterium Aphanizomenon gracile in northeast Germany.* Appl Environ Microbiol, 2010. **76**(4): p. 1173-80.

19. Hoeger, S.J., et al., *Occurrence and elimination of cyanobacterial toxins in two Australian drinking water treatment plants.* Toxicon, 2004. **43**(6): p. 639-649.

20. Orr, P.T., Jones, G.J., and Hamilton, G.R., *Removal of saxitoxins from drinking water by granular activated carbon, ozone and hydrogen peroxide-implications for compliance with the Australian drinking water guidelines.* Water Res, 2004. **38**(20): p. 4455-61.

21. Dixon, M.B., et al., *A coagulation-powdered activated carbon-ultrafiltration - Multiple barrier approach for removing toxins from two Australian cyanobacterial blooms.* Journal of Hazardous Materials, 2011. **186**(2,Äì3): p. 1553-1559.

22. ADWG, *Australian Drinking Water Guidelines Paper 6 National Water Quality Management Strategy.* 2011(National Health and Medical Research

Council, National Resource Management Ministerial Council, Commonwealth of Australia, Canberra).

23. Burch, M., *Effective doses, guidelines & regulations*, in *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs*, H.K. Hudnell, Editor. 2008, Springer New York. p. 831-853.

24. Zegura, B., Straser, A., and Filipic, M., *Genotoxicity and potential carcinogenicity of cyanobacterial toxins - a review.* Mutat Res, 2011. **727**(1-2): p. 16-41.

25. Jones, G.J. and Negri, A.P., *Persistence and degradation of cyanobacterial paralytic shellfish poisons (PSPs) in freshwaters.* Water Research, 1997. **31**(3): p. 525-533.

26. Llewellyn, L.E., *The Behavior of Mixtures of Paralytic Shellfish Toxins in Competitive Binding Assays.* Chemical Research in Toxicology, 2006. **19**(5): p. 661-667.

27. Markensten, H., Moore, K., and Persson, I., *Simulated lake phytoplankton composition shifts toward cyanobacteria dominance in a future warmer climate.* Ecol Appl, 2010. **20**(3): p. 752-67.

28. Carey, C.C., et al., *Eco-physiological adaptations that favour freshwater cyanobacteria in a changing climate.* Water Res, 2012. **46**(5): p. 1394-407.

29. Elliott, J.A., *Is the future blue-green? A review of the current model predictions of how climate change could affect pelagic freshwater cyanobacteria.* Water Res, 2012. **46**(5): p. 1364-71.

30. Brackenbury, W.J., et al., *Functional reciprocity between Na+ channel Nav1.6 and beta1 subunits in the coordinated regulation of excitability and neurite outgrowth.* Proc Natl Acad Sci U S A, 2010. **107**(5): p. 2283-8.

31. Jarecki, J. and Keshishian, H., *Role of neural activity during synaptogenesis in Drosophila.* The Journal of Neuroscience, 1995. **15**(12): p. 8177-8190.

32. O'Neill, K., Musgrave, I.F., and Humpage, A., *Extended Low-Dose Exposure to Saxitoxin Inhibits Neurite Outgrowth in Model Neuronal Cells.* Basic & Clinical Pharmacology & Toxicology, 2016: p. n/a-n/a.

33. Grandjean, P. and Landrigan, P.J., *Neurobehavioural effects of developmental toxicity.* Lancet Neurol, 2014. **13**(3): p. 330-8.

34. Reid, K.J., et al., *Undifferentiated murine embryonic stem cells used to model the effects of the blue-green algal toxin cylindrospermopsin on preimplantation embryonic cell proliferation.* Toxicon, 2015. **106**: p. 79-88.

35. Baek, D.H., et al., *Embryotoxicity assessment of developmental neurotoxicants using a neuronal endpoint in the embryonic stem cell test.* Journal of Applied Toxicology, 2012. **32**(8): p. 617-626.

36. Visan, A., et al., *Neural differentiation of mouse embryonic stem cells as a tool to assess developmental neurotoxicity in vitro.* NeuroToxicology, 2012(0).

37. Bain, G., et al., *Embryonic stem cells express neuronal properties in vitro.* Dev Biol, 1995. **168**(2): p. 342-57.

38. Strübing, C., et al., *Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons.* Mechanisms of Development, 1995. **53**(2): p. 275-287.

39. Yamada, M., et al., *Electrical Stimulation Modulates Fate Determination of Differentiating Embryonic Stem Cells.* STEM CELLS, 2007. **25**(3): p. 562-570.

40. Abe, K., et al., *Endoderm-specific gene expression in embryonic stem cells differentiated to embryoid bodies.* Exp Cell Res, 1996. **229**(1): p. 27-34.

41. Rathjen, J., et al., *Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors.* Journal of Cell Science, 1999. **112**(5): p. 601-612.

42. GWRC, *International Guidance Manual for the Management of Toxic Cyanobacteria.* 2009.

43. Nakamizo, T., et al., *Protection of cultured spinal motor neurons by estradiol.* Neuroreport, 2000. **11**(16): p. 3493-7.

44. Mattila, P.K. and Lappalainen, P., *Filopodia: molecular architecture and cellular functions.* Nat Rev Mol Cell Biol, 2008. **9**(6): p. 446-454.

45. Jin, G.P., et al., *Stem cell pluripotency and transcription factor Oct4.* Cell Res, 2002. **12**(5-6): p. 321-329.

46. Fuhrmann, G., et al., *Mouse germline restriction of Oct4 expression by germ cell nuclear factor.* Dev Cell, 2001. **1**(3): p. 377-87.

47. Okada, Y., et al., *Retinoic-acid-concentration-dependent acquisition of neural cell identity during in vitro differentiation of mouse embryonic stem cells.* Developmental Biology, 2004. **275**(1): p. 124-142.

48. Mohn, D., et al., *Mouse Mix gene is activated early during differentiation of ES and F9 stem cells and induces endoderm in frog embryos.* Developmental Dynamics, 2003. **226**(3): p. 446-459.

49. Ng, E.S., et al., *The primitive streak gene Mixl1 is required for efficient haematopoiesis and BMP4-induced ventral mesoderm patterning in differentiating.* Development, 2005. **132**(5): p. 873-884.

50. Pearce, J.J.H. and Evans, M.J., *Mml, a mouse Mix-like gene expressed in the primitive streak.* Mechanisms of Development, 1999. **87**(1–2): p. 189-192.

51. Michalczyk, K. and Ziman, M., *Nestin structure and predicted function in cellular cytoskeletal organisation.* Histol Histopathol, 2005. **20**(2): p. 665-71.

52. Guan, K., et al., *Embryonic stem cell-derived neurogenesis.* Cell and Tissue Research, 2001. **305**(2): p. 171-176.

53. Kawasaki, H., et al., *Induction of Midbrain Dopaminergic Neurons from ES Cells by Stromal Cell–Derived Inducing Activity.* Neuron, 2000. **28**(1): p. 31-40.

54. Mariani, M., et al., *Class III β-tubulin in normal and cancer tissues.* Gene, 2015. **563**(2): p. 109-114.

55. Tischfield, Max A. and Engle, Elizabeth C., *Distinct α- and β-tubulin isotypes are required for the positioning, differentiation and survival of neurons: new support for the 'multi-tubulin' hypothesis.* Bioscience Reports, 2010. **30**(5): p. 319-330.

56. Johnson, G.V.W. and Jope, R.S., *The role of microtubule-associated protein 2 (MAP-2) in neuronal growth, plasticity, and degeneration.* Journal of Neuroscience Research, 1992. **33**(4): p. 505-512.

57. Zhang, J., et al., *Endogenously EGFP-Labeled Mouse Embryonic Stem Cells.* Aging and Disease, 2011. **2**(1): p. 18-29.

58. Fraichard, A., et al., *In vitro differentiation of embryonic stem cells into glial cells and functional neurons.* J Cell Sci, 1995. **108 (Pt 10)**: p. 3181-8.

59. Spitzer, N.C., *Electrical activity in early neuronal development.* Nature, 2006. **444**(7120): p. 707-12.

Conclusion

The work presented here has drawn attention to low dose extended exposure to saxitoxin and its analogues, to our knowledge the first research of its kind. In chapter one we have reviewed the currently available literature, highlighting the lack of research into and the potential complications of this pattern of exposure which could be of significant concern to public health given the action of STX at multiple ion channels and the role of ion channels and their associated electrical activity in neurodevelopment.

In chapter 2 we have shown that exposure to STX at or below the drinking water guideline caused significant concentration dependent decreases in the development of neuronal morphology following extended exposure.

In chapter 3 we have established an assay to determine if the adverse neurodevelopmental effects seen in chapter one were a result of direct STX toxicity. We found that STX did not cause toxicity, eliminating any non-specific cell toxicity as the cause for the adverse effects seen at the concentrations used in our research.

In chapter 4 we moved to a model which more accurately models mammalian neuronal differentiation. We found that STX at the drinking water guideline again decreases the development of neuronal morphology, confirming the results of chapter one and altered the expression of neuronal markers.

Whilst the work presented here does raise awareness on the lack of research into extended exposure to STX, further work is still required.

Investigation of the mechanisms at play is required to determine if it is the well-known channel blocking activity of STX which has caused the effects seen here, if there are downstream effects of this channel blockage or if an unrelated mechanism is responsible.

Further, as STX belongs to a group of structurally related toxins investigation into these analogues would be highly beneficial as the analogues have varying levels of toxicity and it needs to be confirmed if the adverse effects seen here would occur following exposure to a mixture of the analogues which is how exposure occurs in a real world scenario.

In Vivo experiments are also required to established if the adverse effects recorded in cell models are also seen in whole organisms when toxins are not directly applied to cells but instead are subject to the toxicokinetics of an animal.

In conclusion, the work presented here provides a contribution of new knowledge to the fields of environmental toxicology and public health and raises awareness that this acutely toxic substance cannot be ignored at low doses in drinking water or in shellfish.

Not only is this body of work significant for being this first of its kind, it is significant to public health given that exposure to STX can occur globally from both freshwater and marine sources and is likely to increase with predicted future climate changes. These predicted future climatic changes such as increased water temperatures, nutrient loading and stratification as well as altered hydrology will favour not only freshwater cyanobacterial

growth but also marine, giving them a competitive advantage over other phytoplankton.

Appendix 1

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Review or Mini-review

Low dose extended exposure to saxitoxin and its potential neurodevelopmental effects: A review

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ABSTRACT

Saxitoxin (STX) and its analogs, the paralytic shellfish toxins (PSTs), are a group of potent neurotoxins well known for their role in acute paralytic poisoning by preventing the generation of action potentials in neuronal cells. They are found in both marine and freshwater environments globally and although acute exposure from the former has previously received more attention, low dose extended exposure from both sources is possible and to date has not been investigated. Given the known role of cellular electrical activity in neurodevelopment this pattern of exposure may be a significant public health concern. Additionally, the presence of PSTs is likely to be an ongoing and possibly increasing problem in the future. This review examines the neurodevelopmental toxicity of STX, the risk of extended or repeated exposure to doses with neurodevelopmental effects, the potential implications of this exposure and briefly, the steps taken and difficulties faced in preventing exposure.

Contents

Abbriviations: STX, Saxitoxin; PST, Paralytic Shellfish Toxin; PSP, paralytic shellfish poisoning; VGSC, voltage-gated-sodium channel; GTX, Gonyautoxins; EFSA, European Food Safety Authority; hERG, human ether-a-go-go; TTX, Tetrodotoxin; LPS, lipopolysacharride; SOD, superoxide dismutase; GPx, glutathione peroxidase; EROD, ethoxyresorufin-0-deethylase; PROD, penthoxyresorufin-0-deethylase; PAC, powdered activated carbon.

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1. Introduction

STX is a neurotoxin most commonly known for its role in paralytic shellfish poisoning (PSP) and the majority of past research has been focused on acute exposure from this source. However, there is also the potential of extended exposure to low doses of the toxin, from this source and others, and this pattern of exposure has not been thoroughly investigated. While exposure to high doses of STX can be fatal, low dose extended exposure has the potential to affect neurodevelopment through the action of the toxin at voltage-gated sodium channels (VGSCs) which have been shown to play an important role in a developing nervous system.

Low dose extended exposure from shellfish may occur in communities which rely heavily on a seafood diet, consuming more than the daily average and for considerable periods of time. Additionally at risk are small isolated coastal communities who may harvest untested shellfish. It has been shown that tolerance can occur in some populations (Kuiper-Goodman et al., 1999) so that communities harvesting untested shellfish may be exposed to concentrations higher than safety guidelines, which would cause acute poisoning in a sensitive individual, but would go unnoticed in a tolerant individual. In such cases, while acute poisonings may not occur more subtle low dose adverse effects may be taking place.

The toxin is also produced at lower concentrations by freshwater cyanobacteria which can be found in fresh water sources from which drinking water is sourced (Hoeger et al., 2004). Based on human data from acute paralytic shellfish poisoning events, a drinking water guideline value of 3 μg/L has been established in multiple countries including Australia, Brazil and New Zealand (Burch, 2008; ADWG, 2011) and there have been no acute poisonings to date (Zegura et al., 2011).

There are multiple water treatment methods available for the removal of the cyanobacterial cells responsible for the production of STX and the extracellular dissolved toxin (Hoeger et al., 2004). The percentage of each removed depends on the methods used and while consumers are protected from acute toxicity, low dose exposure can still occur and could occur for extended periods of time considering the duration of algal blooms. Although extended low dose extended exposure is more likely via drinking water there is no guideline for long-term exposure as there has been no research into this pattern of exposure.

It has been suggested that the predicted future climatic changes of global warming such as increased water temperatures, nutrient loading and stratification as well as altered hydrology will favor freshwater cyanobacterial growth and give cyanobacteria a competitive advantage over other phytoplankton. In fact harmful algal blooms in marine settings have already been seen to increase since the 1970s (Hallegraeff, 1993; Van Dolah, 2000) and an increase in total cyanobacteria numbers and individual algal bloom durations has been noted since the 1980s (Croome et al., 2011). Additionally the link between algal blooms and eutrophication has been noted since the 1980s (Anderson et al., 2002).

2. STX and its analogs

STX itself is part of a large group of analogs collectively known as the paralytic shellfish toxins (PSTs) or in some cases the saxitoxins. This group has a long history with human poisonings dating back to at least 1793 (Price et al., 1991). Despite this history the toxin was not isolated until 1957 from the butter clam *Saxidomus giganteus*, after which the toxin is named (Schantz et al., 1957). Due to its noncrystalline and highly polar nature, the structure of the toxin was not determined for almost another 20 years (Schantz et al., 1975). STX is one of the most potent natural toxins known, with a place on Schedule 1 of the Chemical Weapons Convention (Llewellyn, 2006b).

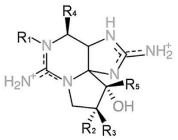

Fig. 1. The tetrahydropurine skeleton of STX and its analogs, for R group substituents see Table 1.

The PST analogs all share a 3,4,6-trialkyl tetrahydropurine skeleton with two guanidinium groups (Schantz et al., 1975) (Fig. 1). Variations to the side chains give the analogs varying levels of toxicity and the analogs are grouped depending on their side chain variations. STX and neoSTX are non-sulfated, the Gonyautoxins (GTXs) mono-sulfated and the C-toxins di-sulfated, each respectively less toxic than STX. Further variants include decarbamoyls. Authors have described up to 57 analogs (Wiese et al., 2010), with the most common shown in Table 1. STX is highly polar and stable in solution (Schantz et al., 1957) while the c-toxins and GTXs are not particularly stable and can degrade to produce more toxic analogs (Fanger et al., 1995). So while the concentration of individual analogs will vary the group of toxins can persist in water for long periods of time, therefore there is a potential for extended exposure periods.

2.1. Sources of STX exposure

As mentioned, STX and its analogs are produced in both marine and freshwater environments. It was originally thought that both marine dinoflagellates and freshwater cyanobacteria produce PSTs by the same biosynthetic pathway (Shimizu, 1993), which is mediated by the *stx* gene cluster in cyanobacteria (Kellmann et al., 2008; Mihali et al., 2009) but the genes responsible for toxin production in dinoflagellates are now thought to be quite different (Yang et al., 2010). It has been recently shown that only a small number of the proteins involved in the biosynthetic pathway in cyanobacteria are present in dinoflagellates, so that the later steps in the pathway may be performed by different reactions or enzymes (Hackett et al., 2013).

The reason why either of these organisms produce the toxin is unknown although there are theories, the most common being defense but from what is not known. Another theory suggests a relationship between intracellular Na^+ levels and STX production, where toxic strains of cyanobacteria would be at an advantage under conditions of high pH or Na^+ stress (Pomati et al., 2004a,b). Based on genetic analysis it has been suggested that the *stx* gene cluster could have emerged at least 2100 Ma, in an environment significantly different to today. At that time organisms had not evolved VGSCs, the most well known target of the PSTs, and so another theory is that the evolutionary predecessor of the channel, the potassium channels, could have been the target of the toxin (Murray et al., 2011).

2.1.1. Marine production of STX

The most well-known and researched source of the STXs are the marine dinoflagellates from the genera *Alexandrium, Gymnodinium* and *Pyrodinium* (Harada et al., 1982; Lefebvre et al., 2008; Oshima et al., 1987). The marine dinoflagellates produce PSTs which are consumed by invertebrates such as shellfish, crustaceans and molluscs, and rarely fish (Deeds et al., 2008). The majority of

Table 1
Side chain variations to the tetrahydropurine skeleton for STX and its analogs.

Toxin	R1	R2	R3	R4	R5	Mouse Bioassay[a]
STX	H	H	H	$OCONH_2$	OH	1656–2483
neoSTX	OH	H	H	$OCONH_2$	OH	1038–2300
Gonyuatoxins						
GTX1	OH	H	OSO_3^-	$OCONH_2$	OH	1638–2468
GTX2	H	H	OSO_3^-	$OCONH_2$	OH	793–1028
GTX3	H	OSO_3^-	H	$OCONH_2$	OH	1463–2234
GTX4	OH	OSO_3^-	H	$OCONH_2$	OH	1803
GTX5	H	H	H	$OCONHSO_3^-$	OH	160
GTX6	OH	H	H	$OCONHSO_3^-$	OH	Not done
C-toxins						
C1	H	H	OSO_3^-	$OCONHSO_3^-$	OH	15–17
C2	H	OSO_3^-	H	$OCONHSO_3^-$	OH	237–329
C3	OH	H	OSO_3^-	$OCONHSO_3^-$	OH	33
C4	OH	OSO_3^-	H	$OCONHSO_3^-$	OH	143
Decarbamoylated						
dcSTX	H	H	H	OH	OH	955–1274
dcneoSTX	OH	H	H	OH	OH	Not done
dcGTX1	OH	H	OSO_3^-	OH	OH	Not done
dcGTX2	H	H	OSO_3^-	OH	OH	1617
dcGTX3	H	OSO_3^-	H	OH	OH	1872
dcGTX4	OH	OSO_3^-	H	OH	OH	Not done

Adapted from (Llewellyn, 2006b; Wiese et al., 2010).
[a] Mouse units/μmol where 1 mouse unit is the amount of toxin what kills a 20 g mouse in 15 min.

these vectors are not affected by the toxin. The toxins become concentrated by these vectors and are ingested by shellfish consumers, causing PSP (Wiese et al., 2010). There are strict regulatory limits for this source of exposure, a maximum of 80 μg STXequivalents/100 g of shellfish tissue, and there are prevention programs set up globally as the number of countries affected by harmful algal blooms (HAB) containing PSTs increases (Etheridge, 2010; Van Dolah, 2000). This regulatory limit was first established in the 1930s, originally in Mouse Units, before the structure and toxicology of the toxins were fully understood, but has been effective in protecting seafood consumers since (Wekell et al., 2004). The vast majority of poisonings in recent times have been a result of harvesting shellfish from quarantined areas, knowingly or unknowingly, or from harvesting untested shellfish which could be a problem for small isolated coastal communities. More recently acute exposure to the PSTs has been reassessed by the European Food Safety Authority EFSA (2009) and a guideline of 7.5 μg STXeq/100 g has been suggested, considerably less than the regulatory limit stated above.

Table 2
Locations and characteristics of freshwater PST production. [1]Humpage et al., 1994) [2]Lagos et al., 1999 [3]Mahmood and Carmichael (1986) [4]Carmichael et al. (1997) [5]Pereira et al. (2000) [6]Ferreira et al. (2001) [7]Pereira et al. (2004) [8]Ballot et al. (2010) [9]Pomati et al. (2000) [10]Liu et al. (2006a) [11]Liu et al. (2006b).

Country	Toxic species	PST analogs produced
Australia	*Dolichospermum*[1]	C1, C2, GTX2, GTX3 and STX
Brazil	*Cylindrospermopsis raciborskii*[2]	neoSTX and STX
USA	*Aphanizomenon flos-aquae*[3]	neoSTX and STX
	Lyngbya wollei[4]	dc-GTX2, dc-GTX3, dcSTX and 6 unidentified analogs
Portugal	*Aphanizomenon flos-aquae*[5,6]	neoSTX, dcSTX, STX, GTX6, GTX5,
	Aphanizomenon gracile[7]	GTX4, GTX1, GTX3
Germany	*Aphanizomenon gracile*[8]	neoSTX and STX
Italy	*Planktothrix* sp.[9]	GTX5, STX, dcSTX and neoSTX
China	*Aphanizomenon flos-aquae*[10,11]	STX
		STX, neoSTX, GTX5, dcSTX and dcGTX3

2.1.2. Freshwater production of STX

STX and its analogs are also produced by freshwater cyanobacteria including those in the genera *Dolichospermum* (previously *Anabaenea* (Wacklin et al., 2009)), *Cylindrospermopsis, Aphanizomenon, Plankotothrix* and *Lyngbya* (Carmichael et al., 1997; Humpage et al., 1994; Lagos et al., 1999; Mahmood and Carmichael, 1986; Onodera et al., 1997; Pereira et al., 2000; Pomati et al., 2000). Their presence in freshwater has been recorded globally with different genera being responsible in each country. There are toxic and non-toxic sub populations of these cyanobacteria and there is additional variation in which toxic analogs each produce (Table 2).

With seasonal changes in cell numbers, the toxin concentration can vary in fresh water bodies throughout the year and between water sources (<1- approx. 23 μg/L raw water, 5–3400 μg/g dry weight) (Hoeger et al., 2004; Kuiper-Goodman et al., 1999). The toxins can persist in water for several months and analogs can undergo transformation to become more toxic, so that concentrations of individual analogs continuously vary (Jones and Negri, 1997). Even when there is a predominance of less toxic analogs, the toxicity of a mixture of these toxins is still largely defined by the lower concentrations of more toxic analogs (Llewellyn, 2006a).

PSP producing cyanobacteria have been shown to carry the *stx* gene cluster. It is suggested that the *stx* gene cluster has a single origin amongst cyanobacteria because only a small number of genes have recombinations between species (Mihali et al., 2009), and that this strong conservation of the stx gene cluster over time shows it has a vital role in the survival of the species that possess it (Murray et al., 2011).

As mentioned above the predicted future climatic changes of global warming will favor the growth of cyanobacteria. These favored growth conditions could have implications for the frequency, timing, size, duration and distribution of algal blooms, with the general consensus that these will all increase (Carey et al., 2012; Elliott, 2012; Jones et al., 2011; Markensten et al., 2010). While the effect this will have on toxin production is still unclear it is predicted that toxin production will increase as growth conditions are

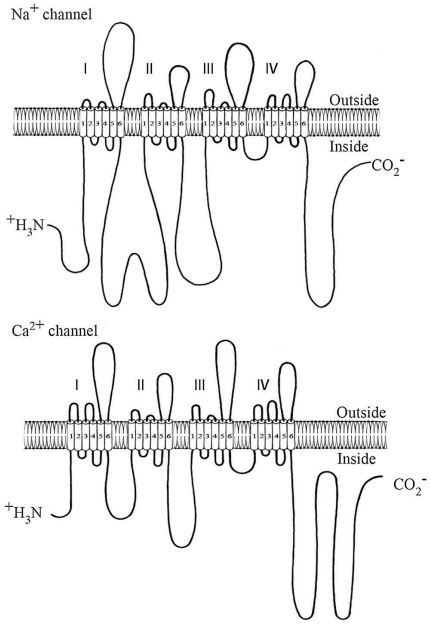

Fig. 2. Arrangement of the sodium and calcium channel α-subunit, showing the 4 identical domains each with 6 transmembrane segments, redrawn from Catterall (1988).

more favorable (Reichwaldt and Ghadouani, 2012) which will subsequently alter the concentrations of toxin reaching drinking water and the patterns of exposure to toxin, having potentially serious consequences for drinking water (Paerl and Paul, 2012).

2.2. STX activity

The most well-known action of STX is its ability to block VGSCs, but it has also been described to act at calcium (Jones and Marks, 1989; Su et al., 2004) and human ether-a-go-go (hERG) potassium channels (Wang et al., 2003). Furthermore it binds to the protein saxiphilin, a transferrin-like protein originally isolated from the North American bullfrog, *Rana castesbeiana* (Llewellyn et al., 1997) as well as a pufferfish protein isolated from *Fugu pardalis* (Yotsu-Yamashita et al., 2001).

2.2.1. Activity at sodium channels

The VGSCs are transmembrane proteins made up of an α-subunit and one or more β-subunits. The α-subunit is made up of 4 identical domains each with 6 transmembrane segments (Sato et al., 2001) (Fig. 2). The domains are arranged around the central pore of the channel with segment 6 forming the inner surface. STX binds to Site 1, which is located on the hairpin-like pore loop (P-loop) between segments 5 and 6 (Stevens et al., 2011), blocking the pore of the channel and preventing the flow of Na ions (Savio-Galimberti et al., 2012; Wiese et al., 2010) with the gating of VGSC having no effect on the blocking action of STX. It has been suggested that there are two predominantly negatively charged rings of carboxylated amino acids within the proximity of the channel pore on the P-loop and this is where the positively charged guanidinium groups of STX bind, with the IC_{50} shown to be 1.2 ± 0.2 nM in wild type rat sodium channel type 2 (Terlau et al., 1991). Alterations such as the addition of a hydroxyl group or a sulfate group can change the affinity of the toxin for the channel giving the different analogs their varying toxicity (Llewellyn, 2006b).

Table 3
Spatial and temporal expression of the VGSC isoforms. CNS = central nervous system, PNS = peripheral nervous system [1] Shafer et al. (2005) [2] Boiko et al. (2001) [3] Shinohara et al. (2011) [4] Benn et al. (2001).

Isoform	Spatial expression[1]	Temporal expression
$Na_v1.1$	CNS	Postnatal to adult nervous tissue[1]
$Na_v1.2$	CNS	Embryonic to adult nervous tissue[1,2]
$Na_v1.3$	CNS	Embryonic to adult nervous tissue[1]
Nav1.4	Skeletal muscle	Postnatal to adult cardiac tissue[3]
Nav1.5	Cardiac muscle	Embryonic to Adult cardiac tissue[1,3]
Nav1.6	CNS	Adult nervous system[2]
Nav1.7	PNS	Adult nervous system[1]
Nav1.8	PNS	Embryonic to adult nerve cells[4]
Nav1.9	PNS	Embryonic to adult nerve cells[4]

Ten different isoforms of the α-subunit have been described ($Na_v1.1$ to $Na_v1.9$) each with varying distribution throughout the human body and each with varying sensitivity to STX (Gilchrist and Bosmans, 2012; Shinohara et al., 2011; Walker et al., 2012).

Isoforms $Na_v1.1$, $Na_v1.2$, $Na_v1.3$ and $Na_v1.6$ are found mainly in the central nervous system, while $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ are found in the peripheral nervous system. These groups of isoforms are known as 'brain type' or 'neuronal-type' sodium channels. $Na_v1.4$ and $Na_v1.5$ are both non-neuronal with $Na_v1.4$ mainly found in skeletal muscle and $Na_v1.5$ being cardiac-exclusive (Savio-Galimberti et al., 2012). Hence different regions of the body will have varying sensitivity to the PSTs. Isoform expression additionally varies during development so that sensitivity to the PSTs may vary over time (Shafer et al., 2005) (Table 3).

While all of the α-subunit isoforms have been classified as either sensitive, resistant or insensitive to tetrodotoxin (TTX), a structurally different toxin which also acts on VGSC, this has not been done for all isoforms in regards to the PSTs. Testing the sensitivity of each isoform would be beneficial to better understanding which regions of the body would be more at risk to extended low dose exposure to STX and during which stages of life.

The β-subunits, which can be found in excitable and non-excitable cells in both the central and peripheral nervous system, are made up of an extracellular domain, a transmembrane domain and an intracellular domain (Savio-Galimberti et al., 2012; Patino and Isom, 2010). Each α-subunit is can be associated with one covalently linked β-subunit (β2 or β4) and one non-covalently linked β-subunit (β1 or β3). The β-subunits have a role as regulatory proteins which can modulate the expression of VGSC on the cell surface, and can modulate cellular migration as well as neurite extension (Patino and Isom, 2010). As mentioned STX binds to the α-subunit and is not known to have any direct effect on the β-subunit.

The primary function of VGSC is to produce action potentials. Neurons and other excitable cells have an electrical potential between the intra- and extracellular environments caused by an imbalance of ions across the cellular membrane. By eliminating and reestablishing this potential, neurons can transmit electrical signals along their axons. Removal of the potential due to certain stimuli opens the VGSC allowing an inflow of Na^+ ions rapidly depolarizing the cell, closure of the channel then allows for repolarization by the oubaine sensitive Na/K antiporter and a return to steady state completing the action potential. By blocking the movement of Na ions

through the channel, STX halts the generation of action potentials and the neurons lose their ability to transmit electrical impulses.

2.2.2. Activity at calcium channels

Voltage-gated calcium channels are also transmembrane proteins and can be made up of four components, α_1-subunit, β-subunit, $\alpha_2\delta$-subunit and calmodulin (Findeisen and Minor, 2010). The α_1-subunit forms the channel pore similarly to the VGSC, having 4 homologous domains each with 6 transmembrane segments and having a similar amino-acid sequence (Tanabe et al., 1987) (Fig. 2). Again there are multiple isoforms of this channel with varying distribution and sensitivity to STX. The high-voltage activated channels including the L-, P/Q- and R-type are made up of all four components mentioned above whereas the T-type which is low-voltage activated needs only the α_1-subunit to function (Findeisen and Minor, 2010). N- and P-type calcium channels are mainly neuronal, with T- and L-type channels being muscular and neuronal (Llewellyn, 2006a,b).

It has been shown that STX blocks calcium currents in bullfrog sympathetic neurons expressing N-type channels with an IC_{50} of 400 nm (Jones and Marks, 1989) and more recently it was shown to partially block L-type calcium channels with an IC_{50} of 0.3 μM (Su et al., 2004 It has been suggested that STX's binding site on this channel is external and may be similar to the VGSC b).inding site (Su et al., 2004). This suggested binding site is not surprising considering the similarities in the α_1 −subunit of the calcium channel and the α_1 −subunit of the VGSC. This same group demonstrated that greater concentrations of STX were required for the same level of channel blockade in calcium channels as in neuronal VGSCs, but lower than those required for cardiac VSGCs.

The channel is responsible for control of cellular calcium entry and has a role in multiple cellular functions including, but not limited to, muscle contraction, neurotransmitter and hormone release and calcium-dependent gene regulation (Findeisen and Minor, 2010; Tanabe et al., 1987).

2.2.3. Activity at potassium channels

Also a transmembrane channel, the hERG K^+ channel is made up of four α-subunits each with six transmembrane segments (S1-S6) (Hari Narayana Moorthy et al., 2013). S1-S4 of each α-subunit make up the voltage sensing domain surrounding S5-S6 which make up the central pore domain (Wang et al., 2003). The hERG K^+ channel has small structural differences from the majority of other voltage gated K^+ channels which could explain why PSPs have only been seen to bind hERG K+ channels (Du et al., 2007).

Mainly found in cardiac myocytes and neurons, the hERG K^+ channel controls the duration of cardiac action potentials through repolarization and has been linked to syndromes which can cause fatal cardiac arrhythmias (Curran et al., 1995; Sanguinetti et al., 1995; Schönherr et al., 1999). The channel has also been detected in human pancreatic β-cells (Rosati et al., 2000). Unlike in VGSCs, STX did not act by blocking the ion pore of this channel, but instead modified the gating of it in HEK-293 cells. Stronger depolarization was required to open channels bound with STX and upon repolarization the channels closed much faster (Wang et al., 2003). Modification of the channel was membrane potential dependent, at −50 mV EC50 was 5.6 μM and at 50 mV EC50 was 0.3 μM.

2.3. Exposure to STX

2.3.1. Acute exposure to STX

Acute exposure to STX occurs as a result of eating contaminated shellfish, potentially resulting in PSP, or from ingestion of contaminated freshwater. As mentioned above no acute human poisonings have been recorded from freshwater, yet there have been livestock deaths (McBarron et al., 1975; Negri et al., 1995). However, close to 2000 PSP cases are reported yearly around the world with a fatality rate of 15% (Hallegraeff, 1993) and fatalities have been recorded in marine life also (Reyero et al., 1999).

Following exposure to STX, action potentials in nerve and muscle fibers are interrupted. Symptoms can be both gastrointestinal and neurological including; paraesthesia, nausea, vomiting, incoordination, diarrhea, weakness, ataxia, shortness of breath, dysarthria, dysphagia, hypotension and, depending on the amount of toxin consumed, can result in complete paralysis of the victim and death via respiratory depression (de Carvalho et al., 1998; Etheridge 2010). The outcome of PSP is variable between individuals, for example children have been shown to be more susceptible with a higher mortality rate (de Carvalho et al., 1998; Gessner et al., 1997; Rodrigue et al., 1990).

Death can occur within an hour of a lethal dose and there is currently no antidote, with the only sufficient treatment being artificial respiration (Llewellyn, 2006b,a). PSP victims may also be treated with activated charcoal to remove any unabsorbed toxin (Pearson et al., 2010). Victims surviving more than 24 h have much greater chances of making a full recovery (Long et al., 1990).

It has also been shown that PSTs can be bioaccumulated in the freshwater environment in the same fashion as the marine source, but with different vectors. The filter-feeding freshwater mussel *Alathyria condola* Iredale has been shown to accumulate PSTs when fed *A. circinalis* for 7 days and extracts from mussels produced typical PSP symptoms in the mouse bioassay (Negri and Jones, 1995). Whilst these mussels are not commonly consumed, they are occasionally consumed by populations of Indigenous Australians and they are consumed by aquatic animals.

Exposure to PSTs can also occur during recreational water use. In addition to the PSTs cyanobacteria are also known to produce lipopolysaccharide (LPS) endotoxins (Papageorgiou et al., 2004) so that during recreational exposure adverse reactions may be a result of the PSTs and/or LPS. In a large international cohort study the majority of symptoms recorded following recreational exposure to cyanobacteria were mild respiratory, however there was not a significant increase compared to controls (Stewart et al., 2006).

2.3.2. Pharmacokinetics of STX

The effects of exposure to the PSTs obviously depends on the pharmacokinetics of the toxins. Beginning with absorption, GTX 2/3 epimers have been shown to be readily transported across the epithelium by both paracellular and transcellular routes (Andrinolo et al., 2002a, 2002b; Torres et al., 2007 in Caco-2 and IEC-6 cells as well as in human intestinal samples. Analysis of tissue and body fluids taken post-mortem from victims of PSP show distribution of the toxin in the brain, bile, cerebrospinal fluid, liver, spleen, heart, thyroid and adrenal glands, kidneys, pancreas and lungs (Garcia et al., 2004). Pharmacokinetic studies in cats again showed distribution throughout the body in the brain, medulla oblongata, liver and spleen, with the largest percentage of the dose reaching the spleen (Andrinolo et al., 1999). This widespread distribution explains both the neurological and gastrointestinal symptoms resulting from PSP and demonstrates the capability of the PSTs to cross the blood-brain barrier. It is worth noting that these studies used high concentrations of STX which caused death in the cats and the post-mortem analysis were from victims of PSP, therefore receiving high concentrations of toxin also. This questions whether the ability of STX to cross the blood-brain barrier would be seen at low concentrations. Cardiovascular burdens and hypercapnia can significantly alter the permeability of the blood-brain barrier, conditions which have been recorded following exposure to high doses of STX (Chang et al., 1993; Evans et al., 1976), but if these effects are not seen at low doses of STX exposure will the toxin still be able to cross the blood-brain barrier? If not, the toxin will still exert its effect on the peripheral nervous system.

In a more recent study in cats using GTX2/3 epimers it was shown that the toxins are mainly excreted via glomerular filtration (Andrinolo et al., 1999) which supports the earlier work in cats and rats which suggested excretion through urine (Andrinolo et al., 2002b; Hines et al., 1993). There is some difference in opinion as to whether or not the PSTs are metabolized. In the same pharmacokinetic study mentioned above only GTX2 and GTX3 were recovered in urine and following incubation of GTX2/3 epimers with cat liver supernatant 100% of the toxin was recovered, suggesting that no metabolism took place (Andrinolo et al., 2002a). Similarly the urine of rats treated with dihydrosaxitoxin did not show any other toxin analogs (Hines et al., 1993).

In contrast the post-mortem analysis mentioned above recorded STX and GTX1-5 in the gastric content, yet in the body fluids, spleen and pancreas neoSTX was recorded, as was dcSTX in liver, kidneys and lungs suggesting that transformation of the analogs does occur in humans (Garcia et al., 2004). The same group has shown that GTX2 and GTX3 can undergo oxidation and subsequent glucuronidation by human liver microsomes *in vitro*, converting them to four major metabolites; glucuronic-GTX3, glucuronic-GTX2, GTX4 and GTX1 (García et al., 2009) and have also shown the same conversion of neoSTX and STX to glucuronic-neoSTX and glucuronic-STX respectively (Garcia et al., 2010). In each study they recovered only 6% and 15% of the original PSTs respectively, suggesting that the majority are converted. This glucoronidation metabolic pathway could explain the increased sensitivity to PSTs in children as glucoronidation develops during childhood (Strassburg et al., 2002) and the earlier work which did not record any metabolism of the PSTs in cats is likely to be due to the fact that glucouronidation is not a metabolic pathway found in cats (Wiese et al., 2010).

Metabolism is also supported by findings from another post-mortem analysis of a PSP victim where the toxin profile of the gut varied greatly from that of the urine (Llewellyn et al., 2002). The gut content was made up primarily of STX with lesser amounts of GTX2, GTX3 and neoSTX, whereas in the urine only half of the toxin found was STX; GTX2 and GTX3 were almost completely removed and there was an increase in neoSTX and dcSTX. The group proposed that the N_1 group of STX underwent oxidation for the conversion to neoSTX and hydrolysis of the carbamoyl group for conversion to dcSTX. The different toxin profile seen post-mortem compared to the microsome results could suggest that further metabolism occurs in the kidney or bladder.

As mentioned above the toxicity of a mix of toxins is largely defined by the more toxic analogs even if they are at a lower concentration in comparison to the less toxic analogs (Llewellyn, 2006a). Therefore it is worth noting that the effect of metabolism on the overall toxicity of a mix of PSPs analogs may be quite small.

2.3.3. Chronic exposure to STX

To date there has been little research into the reproductive, teratogenic, genotoxic or carcinogenic effects of the PSTs (Munday and Reeve 2013; Zegura et al., 2011) despite extended low dose exposure being a possibility.

The majority of work that has been done on extended exposure to STXs was carried out in amphibians and fish. It was shown that extended exposure to STX in zebrafish had significant adverse effects on morphology, growth and survival (Lefebvre et al., 2004). Similar morphological effects were seen by another group using zebrafish who also observed an increase in mortality during larval development and altered hatching time, this altered hatching time was also seen in axolotl (Oberemm et al., 1999). Due to the high concentrations used in both experiments (10–500 μg/L STX), it is likely that the effects on growth and survival were a result of paralysis and reduced feeding, which was noted in the treated fish of the first study. Concentrations used in both experiments were much greater than those which people are likely to be exposed to for an extended period so that no suggestions can be made of what would be expected to result in humans, but it does highlight the lack of research into this pattern of exposure.

When exposed to lower concentration of PSTs both fish and mammalian models have shown significant changes to antioxidant mechanisms (Hong et al., 2003) as well as DNA damage suggestive of apoptosis (da Silva et al., 2011). Antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) were significantly reduced in the liver of mice exposed to sub-lethal concentrations of GTX2/3 (300 μg//kg and 200 μg/kg respectively) as were the xenobiotic metabolizing enzymes ethoxyresorufin-*0*-deethylase (EROD) and penthoxyresorufin-*0*-deethylase (PROD).The less toxic analogs C1/2 (450 μg/kg and 1960 μg/kg respectively) also significantly lowered the activity of PROD and GPx (Hong et al., 2003).

Further, rats ingesting STX spiked drinking water (3 μg/L and 9 μg/L in drinking) for 30 days had significant changes to antioxidant mechanisms in both the brain and liver including total antioxidant capacity and levels of reactive oxygen species, lipid hydroperoxides and oxidative enzymes (Ramos et al., 2014). At the drinking water guideline of 3 μg/L there was a reduction in the total antioxidant capacity in the hippocampus but not in the pre-frontal cortex suggesting that not all areas of the brain would be affected equally following extended low-dose exposure to the PSTs.

These changes in antioxidant mechanisms could cause an increase in oxidative stress and have adverse effects on neurodevelopment as the brain has an increased vulnerability to oxidative stress and fewer defenses (Gupta, 2004), especially the developing brain having increased metabolic demand required for growth and fewer glia (Song et al., 2011), which have been shown to protect neurons from oxidative stress (Tanaka et al., 1999). How the PSTs mediate these changes to antioxidant mechanisms is unknown.

By reducing the action of metabolizing enzymes STX may alter the metabolism of other compounds or it may very well reduce the rate of its own metabolism creating the potential for enzyme polymorphisms to result in inter-individual differences in response to STX. This supports the idea that the PSTs may have adverse effects not just in the nervous system.

Non neurological adverse effects have been seen in wild fish following extended exposure to PSTs (4.6–83.44 STX eq ng/L) (Clemente et al., 2010). These included epithelial hyperplasia in the gills as well as necrosis and Melano-macrophage centers in the liver.

Another potential outcome of extended exposure is alterations in neurogenesis as electrical activity has been described as playing an important role in normal CNS development (Brackenbury et al., 2010). Therefore the activity of low concentrations of STX at voltage gated ion channels could have implications for neurodevelopment. For example, disruption of electrical activity using TTX has been shown to inhibit neurite outgrowth mediated by the β1 subunit of the VGSC channel (Brackenbury et al., 2010), triggering neuronal apoptosis mediated by a rise in postsynaptic response to glutamate and reduced ability to clear intracellular calcium (Fishbein and Segal, 2007). In *Drosophila*, an increase in sprouting from the transverse nerve onto muscle fibers at neuromuscular junctions has been shown to occur following treatment with TTX (Jarecki and Keshishian, 1995).Furthermore it has also been shown that STX administration can change levels of neuroactive amino acids in multiple brain regions of rats, yet the particular channel-blocking action of STX causing these changes is unknown (Cianca et al., 2008).

Calcium channels have also been implicated in proper neuronal development. These channels have been shown to have an impor-

tant part in the activity-dependent refinement of connections at neuromuscular junctions in *Drosophila* (Carrillo et al., 2010), suggesting that STX's action at these ion channels could have a further effect on development.

2.4. Measures taken and difficulties faced in preventing exposure

As there is no antidote and limited treatment available for PSP, it is of great importance to prevent exposure. As mentioned there is a guideline of 80 μg STXeq/100 g of shellfish and this has been very successful at preventing acute lethal poisoning from this source, however low dose non-symptomatic exposure can still occur. Given that the assumed daily consumption of shellfish is 200 g this would equate to a total of 160 μg STX eq or 2.7 μg STXeq/kg body weight (b.w) for a 60 kg adult. In comparison drinking water guidelines are 3 μg/L STX and the assumed daily consumption of water is 2L which would equate to a total of 6 μg STXeq or 0.1 μg STXeq/kg b.w for a 60 kg adult making it even more precautionary in preventing exposure. Additionally, as the 2L is an assumed daily consumption and STX does not accumulate in the body the actual concentration of STX an individual is exposed to at any one time is likely to be less than this.

As mentioned above the EFSA has suggested lowering the regulatory guideline for shellfish to 7.5 μg STXeq/100 g of shellfish, based on an estimated no-observed-adverse-effect level of 0.5 μg STXeq/kg b.w. They have also noted the large variation in the amount of shellfish consumed in different populations and that the current guideline for shellfish exposure is not protective enough and should be adjusted to protect people consuming large amounts of shellfish (2009). These communities which consume large amounts of shellfish could be at risk of extended low dose exposure to STX if consumed on a daily basis.

The first approach to preventing PST exposure in drinking water is prevention of algal blooms that produce the toxins. Reservoir mixing or destratification can be used to reduce cyanobacterial growths. Secondly freshwater can successfully be treated so that PST concentrations are below guidelines of 3 μg/L. The best approach is to have a multi barrier method so that both intracellular and extracellular toxins can be removed. As STX is predominantly intracellular, treatments such as coagulation and filtration are effective in removing cells and therefore the majority of toxins, then the addition of powdered activated carbon (PAC) is used to remove extracellular toxins, and chlorination for disinfection. The combination of coagulation, PAC and ultrafiltration has been shown to remove all cyanobacterial cells and 90% of intracellular PSTs, but extracellular PSTs could not be successfully quantified due to their low concentration (Dixon et al., 2011). Other treatments can include granular activated carbon filtration and ozonation (Dixon et al., 2011; Orr et al., 2004).

Although treatments are successful at lowering the concentration of STX below guidelines, low concentrations may still be present in drinking water. For example spot checks following treatment recorded PSPs close to 0.5 μg/L (Hoeger et al., 2004). This same group has shown that PSTs can persist in water for consecutive months.

To guarantee the safety of drinking water, methods used to quantify concentrations of PSTs must be accurate and precise, for which there are numerous techniques each with their own advantages and drawbacks (Humpage et al., 2010). With further research into this pattern of exposure it is also important to have methods available which can accurately and precisely quantitate concentrations of PSTs, if the investigation of low-dose extended exposure to the PSPs resulted in lowering of the guidelines, current analytical methods may not be sensitive enough.

3. Conclusion

The PSTs are a group of potent neurotoxins with a long history of poisoning from marine sources with their production by freshwater cyanobacteria being a more recent discovery. The public health risk of acute exposure via marine sources is well managed with regulatory guidelines and monitoring programs and acute poisoning from freshwater is unlikely due successful water treatment. So while acute PST poisoning is uncommon low dose extended exposure, about which very little is known, is still a public health risk

This pattern of exposure is of particular concern considering the action of STX on multiple ion channels and the role of ion channels and their associated electrical activity in neurodevelopment. It is likely that the PSTs will not just be causing an inhibition of action potentials via their activity at VGSC, but could in fact be preventing more complex cellular pathways orchestrated by these multiple ion channels. Evidence of this has already been seen with significant effects on cellular antioxidant mechanisms.

Additionally, it is likely that production of PSTs will be an ongoing concern as cyanobacteria growth has been predicted to increase with the predicted future changes to climate. This together with the lack of research and possible neurodevelopmental effects of extended exposure makes low dose STX in freshwater a significant public health concern which must be addressed.

Declaration of interest

The authors report no conflicts of interest. The author's affiliation is as shown on the cover page. The authors have sole responsibility for the writing and content of the paper

References

Anderson, D.M., Glibert, P.M., Burkholder, J.M., 2002. Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. Estuaries 25 (4), 704–726, Available from http://www.jstor.org/stable/135302.

Andrinolo, D., Michea, L.F., Lagos, N., 1999. Toxic effects, pharmacokinetics and clearance of saxitoxin, a component of paralytic shellfish poison (PSP), in cats. Toxicon 37 (3), 447–464, Available from http://www.scopus.com/inward/record.url?eid=2-s2.0-0033034468partnerID=40md5=81b40b698ca0c0cf09a713c198aa1c68.

Andrinolo, D., Gomes, P., Fraga, S., Soares-da-Silva, P., Lagos, N., 2002a. Transport of the organic cations gonyautoxin 2/3 epimers, a paralytic shellfish poison toxin, through the human and rat intestinal epitheliums. Toxicon 40 (10), 1389–1397, http://dx.doi.org/10.1016/S0041-0101(02)00118-6, Available from http://www.sciencedirect.com/science/article/pii/S0041010102001186.

Andrinolo, D., Iglesias, V., García, C., Lagos, N., 2002b. Toxicokinetics and toxicodynamics of gonyautoxins after an oral toxin dose in cats. Toxicon 40 (6), 699–709, http://dx.doi.org/10.1016/S0041-0101(01)00263-X, Available from http://www.sciencedirect.com/science/article/pii/S004101010100263X.

Ballot, A., Fastner, J., Wiedner, C., 2010. Paralytic shellfish poisoning toxin-producing cyanobacterium Aphanizomenon gracile in northeast Germany. Appl. Environ. Microbiol. 76 (4), 1173–1180, http://dx.doi.org/10.1128/AEM.02285-09, Available from http://www.ncbi.nlm.nih.gov/pubmed/20048055.

Benn, S.C., Costigan, M., Tate, S., Fitzgerald, M., Woolf, C.J., 2001. Developmental expression of the TTX-Resistant voltage-gated sodium channels nav1.8 (SNS) and nav1.9 (SNS2) in primary sensory neurons. J. Neurosci. 21 (16), 6077–6085, Available from http://www.jneurosci.org/content/21/16/6077.abstract.

Boiko, T., Rasband, M.N., Levinson, S.R., Caldwell, J.H., Mandel, G., Trimmer, J.S., Matthews, G., 2001. Compact myelin dictates the differential targeting of two sodium channel isoforms in the same axon. Neuron 30 (1), 91–104, http://dx.doi.org/10.1016/S0896-6273(01)00265-3, Available from http://dx.doi.org/10.1016/S0896-6273(01)00265-3.

Brackenbury, W.J., Calhoun, J.D., Chen, C., Miyazaki, H., Nukina, N., Oyama, F., Ranscht, B., Isom, L.L., 2010. Functional reciprocity between Na+ channel Nav1.6 and beta1 subunits in the coordinated regulation of excitability and neurite outgrowth. Proc. Natl. Acad. Sci. U. S. A. 107 (5), 2283–2288, Available from http://www.ncbi.nlm.nih.gov/pubmed/20133873 0909434107 [pii] http://dx.doi.org/10.1073/pnas.0909434107.

Burch, M., 2008. Effective Doses, Guidelines & Regulations In: Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs. Springer, New York, pp. 831–853.

Carey, C.C., Ibelings, B.W., Hoffmann, E.P., Hamilton, D.P., Brookes, J.D., 2012. Eco-physiological adaptations that favour freshwater cyanobacteria in a changing climate. Water Res. 46 (5), 1394–1407, http://dx.doi.org/10.1016/j.

watres.2011.12.016, Available from http://www.ncbi.nlm.nih.gov/pubmed/22217430 S0043-1354(11)00788-3 [pii].
Carmichael, W.W., Evans, W.R., Yin, Q.Q., Bell, P., Moczydlowski, E., 1997. Evidence for paralytic shellfish poisons in the freshwater cyanobacterium Lyngbya wollei (Farlow ex Gomont) comb.nov. Appl. Environ. Microbiol. 63 (8), 3104–3110, Available from http://aem.asm.org/content/63/8/3104.abstract.
Carrillo, R.A., Olsen, D.P., Yoon, K.S., Keshishian, H., 2010. Presynaptic activity and CaMKII modulate retrograde semaphorin signaling and synaptic refinement. Neuron 68 (1), 32–44, http://dx.doi.org/10.1016/j.neuron.2010.09.005, Available from http://www.ncbi.nlm.nih.gov/pubmed/20920789 S0896-6273(10)00722-1 [pii].
Catterall, W.A., 1988. Structure and function of voltage-sensitive ion channels. Science 242 (4875), 50–61, http://dx.doi.org/10.2307/1702492, Available from http://www.jstor.org/stable/1702492.
Chang, F.-C.T., Benton, B.J., Lenz, R.A., Capacio, B.R., 1993. Central and peripheral cardio-respiratory effects of saxitoxin (STX) in urethane-anesthetized guinea-pigs. Toxicon 31 (5), 645–664, http://dx.doi.org/10.1016/0041-0101(93)90119-4, Available from http://www.sciencedirect.com/science/article/pii/0041010193901194.
Cianca, R.C.C., RDn, Barbosa, Faro, L.R.F., Adan, L.V., Gago-MartÎnez, A., Pallares, M.A., 2008. Differential changes of neuroactive amino acids in samples obtained from discrete rat brain regions after systemic administration of saxitoxin. Neurochem. Int. 54 (5-6), 308–313, http://dx.doi.org/10.1016/j.neuint.2008.12.014 (Available from http://www.sciencedirect.com/science/article/pii/S0197018608002143.
Clemente, Z., Busato, R.H., Oliveira Ribeiro, C.A., Cestari, M.M., Ramsdorf, W.A., Magalhães, V.F., Wosiack, A.C., Silva de Assis, H.C., 2010. Analyses of paralytic shellfish toxins and biomarkers in a southern Brazilian reservoir. Toxicon 55 (2–3), 396–406, http://dx.doi.org/10.1016/j.toxicon.2009.09.003, Available from http://www.sciencedirect.com/science/article/pii/S0041010109004541.
Croome, R.W.L., Henderson, B., Oliver, R., Vilizzi, L., Paul, W., McInerney, P., 2011. River murray water quality monitoring program: phytoplankton data trend analysis 1980–2008. In: Final Report Prepared for the Murray-Darling Basin Authority by The Murray-Darling Freshwater Research Centre. MDFRC Publication, June. Available.
Curran, M.E., Splawski, I., Timothy, K.W., Vincen, G.M., Green, E.D., Keating, M.T., 1995. A molecular basis for cardiac arrhythmia: HERG mutations cause long QT syndrome. Cell 80 (5), 795–803, http://dx.doi.org/10.1016/0092-8674(95)90358-5, Available from http://www.sciencedirect.com/science/article/pii/0092867495903585.
da Silva, C.A., Oba, E.T., Ramsdorf, W.A., Magalhães, V.F., Cestari, M.M., Oliveira Ribeiro, C.A., Silva de Assis, H.C., 2011. First report about saxitoxins in freshwater fish Hoplias malabaricus through trophic exposure. Toxicon 57 (1), 141–147, http://dx.doi.org/10.1016/j.toxicon.2010.10.015, Available from http://www.sciencedirect.com/science/article/pii/S004101011000396X.
de Carvalho, M., Jacinto, J., Ramos, N., de Oliveira, V., Pinho e Melo, T., de Sa, J., 1998. Paralytic shellfish poisoning: clinical and electrophysiological observations. J. Neurol. 245 (8), 551–554, Available from http://www.ncbi.nlm.nih.gov/pubmed/9747920.
Deeds, J.R., Landsberg, J.H., Etheridge, S.M., Pitcher, G.C., Longan, S.W., 2008. Non-traditional vectors for paralytic shellfish poisoning. Mar Drugs 6 (2), 308–348, http://dx.doi.org/10.3390/md20080015, Available from http://www.ncbi.nlm.nih.gov/pubmed/18728730.
Dixon, M.B., Richard, Y., Ho, L., Chow, C.W.K., O'Neill, B.K., Newcombe, G., 2011. A coagulation-powdered activated carbon-ultrafiltration – Multiple barrier approach for removing toxins from two Australian cyanobacterial blooms. J. Hazard. Mater. 186 (2'Äi3), 1553–1559, http://dx.doi.org/10.1016/j.jhazmat.2010.12.049, Available from http://www.sciencedirect.com/science/article/pii/S0304389410016298.
Du, L., Li, M., You, Q., Xia, L., 2007. A novel structure-based virtual screening model for the hERG channel blockers. Biochem. Biophys. Res. Commun. 355 (4), 889–894, http://dx.doi.org/10.1016/j.bbrc.2007.02.068, Available from http://www.sciencedirect.com/science/article/pii/S0006291×07003191.
EFSA, 2009. Marine biotoxins in shellfish – saxitoxin group scientific opinion of the panel on contaminants in the food chain. EFSA J. 1019, 1–76, Available.
Elliott, J.A., 2012. Is the future blue-green? A review of the current model predictions of how climate change could affect pelagic freshwater cyanobacteria. Water Res. 46 (5), 1364–1371, http://dx.doi.org/10.1016/j.watres.2011.12.018, Available from http://www.ncbi.nlm.nih.gov/pubmed/22244968 S0043-1354(11)00790-1 [pii].
Etheridge, S.M., 2010. Paralytic shellfish poisoning: seafood safety and human health perspectives. Toxicon 56 (2), 108–122, http://dx.doi.org/10.1016/j.toxicon.2009.12.013, Available from http://proxy.library.adelaide.edu.au/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=aph&AN=51147635&site=ehost-live&scope=site.
Evans, C.A., Reynolds, J.M., Reynolds, M.L., Saunders, N.R., 1976. The effect of hypercapnia on a blood-brain barrier mechanism in foetal and new-born sheep. J. Physiol. 255 (3), 701–714, Available from http://www.ncbi.nlm.nih.gov/pubmed/1263141.
Fanger, G.R., Jones, J.R., Maue, R.A., 1995. Differential regulation of neuronal sodium channel expression by endogenous and exogenous tyrosine kinase receptors expressed in rat pheochromocytoma cells. J. Neurosci. 15 (1 Pt 1), 202–213, Available from http://www.ncbi.nlm.nih.gov/pubmed/7823130.
Ferreira, F.M.B., Soler, J.M.F., Fidalgo, M.L., Fernández-Vila, P., 2001. PSP toxins from Aphanizomenon flos-aquae (cyanobacteria) collected in the crestuma-lever reservoir (Douro river, Northern Portugal). Toxicon 39 (6), 757–761, http://dx.doi.org/10.1016/S0041-0101(00)00114-8, Available from http://www.sciencedirect.com/science/article/pii/S0041010100001148.
Findeisen, F., Minor Jr, D.L., 2010. Progress in the structural understanding of voltage-gated calcium channel (CaV) function and modulation. Channels 4 (6), Available from http://www.scopus.com/inward/record.url?eid=2-s2.0-78650842356partnerID=40md5=afa2ab28db0b6d5edda2e6410e85cbca.
Fishbein, I., Segal, M., 2007. Miniature synaptic currents become neurotoxic to chronically silenced neurons. Cereb. Cortex 17 (6), 1292–1306, http://dx.doi.org/10.1093/cercor/bhl037, Available from http://www.ncbi.nlm.nih.gov/ubmed/16835294bhl037 [pii].
García, C., Rodriguez-Navarro, A., Díaz, J.C., Torres, R., Lagos, N., 2009. Evidence of in vitro glucuronidation and enzymatic transformation of paralytic shellfish toxins by healthy human liver microsomes fraction. Toxicon 53 (2), 206–213, http://dx.doi.org/10.1016/j.toxicon.2008.10.028, Available from http://www.sciencedirect.com/science/article/pii/S0041010108005746.
Garcia, C., del Carmen Bravo, M., Lagos, M., Lagos, N., 2004. Paralytic shellfish poisoning: post-mortem analysis of tissue and body fluid samples from human victims in the Patagonia fjords. Toxicon 43 (2), 149–158, http://dx.doi.org/10.1016/j.toxicon.2003.11.018, Available from http://www.sciencedirect.com/science/article/pii/S0041010103003441.
Garcia, C., Barriga, A., Diaz, J.C., Lagos, M., Lagos, N., 2010. Route of metabolization and detoxication of paralytic shellfish toxins in humans. Toxicon 55 (1), 135–144, http://dx.doi.org/10.1016/j.toxicon.2009.07.018, Available from http://www.ncbi.nlm.nih.gov/pubmed/19632259 S0041-0101(09)00368-7 [pii].
Gessner, B.D., Middaugh, J.P., Doucette, G.J., 1997. Paralytic shellfish poisoning in Kodiak, Alaska. West. J. Med. 167 (5), 351–353, Available from http://www.ncbi.nlm.nih.gov/pubmed/9392992.
Gilchrist, J., Bosmans, F., 2012. Animal toxins can alter the function of Nav1.8 and Nav1.9. Toxins (Basel) 4 (8), 620–632, http://dx.doi.org/10.3390/toxins4080620, Available from http://www.ncbi.nlm.nih.gov/ubmed/23012651toxins-04-00620.
Gupta, R.C., 2004. Brain regional heterogeneity and toxicological mechanisms of organophosphates and carbamates. Toxicol. Mech. Methods 14 (3), 103–143, http://dx.doi.org/10.1080/15376520490429175, Available from http://proxy.library.adelaide.edu.au/login?url=http://search.ebscohost.com/login.aspx?direct=truedb=hchAN=12751193site=ehost-livescope=site.
Hackett, J.D., Wisecaver, J.H., Brosnahan, M.L., Kulis, D.M., Anderson, D.M., Bhattacharya, D., Plumley, F.G., Erdner, D.L., 2013. Evolution of saxitoxin synthesis in cyanobacteria and dinoflagellates. Mol. Biol. Evol. 30 (1), 70–78, http://dx.doi.org/10.1093/molbev/mss142, Available from http://mbe.oxfordjournals.org/content/30/1/70.abstract.
Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32 (2), 79–99, http://dx.doi.org/10.2216/i0031-8884-32-2-79.1, Available from http://dx.doi.org/10.2216/i0031-8884-32-2-79.1.
Harada, T., Oshima, Y., Yasumoto, T., 1982. Structures of two paralytic shellfish toxins, gonyautoxins V and VI, isolated from a tropical Dinoflagellate, Pyrodinium bahamensevar.compressa. Agric. Biol. Chem. 46 (7), 1861–1864, Available.
Hari Narayana Moorthy, S.N., Ramos, J., Fernandes, P.A., 2013. Human ether-a-Go-Go-Related gene channel blockers and its structural analysis for drug design. Curr. Drug Targets 14 (1), 102–113, http://dx.doi.org/10.2174/138945013804806460, Available from http://www.ingentaconnect.com/content/ben/cdt/2013/00000014/00000001/art00011.
Hines, H.B., Naseem, S.M., Wannemacher Jr, R.W., 1993. [3H]-Saxitoxinol metabolism and elimination in the rat. Toxicon 31 (7), 905–908, http://dx.doi.org/10.1016/0041-0101(93)90226-9, Available from http://www.sciencedirect.com/science/article/pii/0041010193902269.
Hoeger, S.J., Shaw, G., Hitzfeld, B.C., Dietrich, D.R., 2004. Occurrence and elimination of cyanobacterial toxins in two Australian drinking water treatment plants. Toxicon 43 (6), 639–649, http://dx.doi.org/10.1016/j.toxicon.2004.02.019, Available from http://www.sciencedirect.com/science/article/B6TCS-4C40PY2-1/2/9670b94f18f0f4b1d04238fb8ff1b40d.
Hong, H.-z, Lam, P.K.S., Hsieh, D.P.H., 2003. Interactions of paralytic shellfish toxins with xenobiotic-metabolizing and antioxidant enzymes in rodents. Toxicon 42 (4), 425–431, http://dx.doi.org/10.1016/S0041-0101(03)00175-2, Available from http://www.sciencedirect.com/science/article/pii/S0041010103001752.
Humpage, A.R., Rositano, J., Bretag, A.H., Brown, R., Baker, P.D., Nicholson, B.C., Steffensen, D.A., 1994. Paralytic shellfish poisons from Australian cyanobacterial blooms. Aust. J. Mar. Freshw. Res. 45 (5), 761–771, Available from http://www.scopus.com/inward/record.url?eid=2-s2.0-0028311816&partnerID=40&md5=aa011b081c2d50cbcfb6477640bf483a.
Humpage, A.R., Magalhaes, V.F., Froscio, S.M., 2010. Comparison of analytical tools and biological assays for detection of paralytic shellfish poisoning toxins. Anal. Bioanal. Chem. 397 (5), 1655–1671, http://dx.doi.org/10.1007/s00216-010-3459-4, Available from http://www.ncbi.nlm.nih.gov/pubmed/20101494.
Jarecki, J., Keshishian, H., 1995. Role of neural activity during synaptogenesis in Drosophila. J. Neurosci. 15 (12), 8177–8190, Available from http://www.jneurosci.org/content/15/12/8177.abstract.
Jones, S.W., Marks, T.N., 1989. Calcium currents in bullfrog sympathetic neurons I. Activation kinetics and pharmacology. J. Gen. Physiol. 94 (1), 151–167, Available from http://www.ncbi.nlm.nih.gov/pubmed/2478659.
Jones, G.J., Negri, A.P., 1997. Persistence and degradation of cyanobacterial paralytic shellfish poisons (PSPs) in freshwaters. Water Res. 31 (3), 525–533, http://dx.doi.org/10.1016/S0043-1354(96)00134-0, Available from http://www.sciencedirect.com/science/article/pii/S0043135496001340.

Jones, I.D., Page, T., Alex Elliott, J., Thackeray, S.J., Heathwaite, A., 2011. Increases in lake phytoplankton biomass caused by future climate-driven changes to seasonal river flow. Global Change Biol. 17 (5), 1809–1820, Available from 10.1111/j.1365-2486.2010.02332.x.

Kellmann, R., Mihali, T.K., Jeon, Y.J., Pickford, R., Pomati, F., Neilan, B.A., 2008. Biosynthetic intermediate analysis and functional homology reveal a saxitoxin gene cluster in cyanobacteria. Appl. Environ. Microbiol. 74 (13), 4044–4053, http://dx.doi.org/10.1128/aem.00353-08, Available from http://www.ncbi.nlm.nih.gov/ubmed/18487408 AEM 00353-08 [pii].

Kuiper-Goodman, T.F., Ian, Fitzgerald, J., 1999. Toxic Cyanobacteria in Water: A Guide to Their Public Consequences, Monitoring and Management. E & FN Spon, London.

Lagos, N., Onodera, H., Zagatto, P.A., Andrinolo, D., Azevedo, S.M.F.Q., Oshima, Y., 1999. The first evidence of paralytic shellfish toxins in the freshwater cyanobacterium Cylindrospermopsis raciborskii, isolated from Brazil. Toxicon 37 (10), 1359–1373, http://dx.doi.org/10.1016/S0041-0101(99)00080-X, Available from http://www.sciencedirect.com/science/article/pii/S004101019900080X.

Lefebvre, K.A., Trainer, V.L., Scholz, N.L., 2004. Morphological abnormalities and sensorimotor deficits in larval fish exposed to dissolved saxitoxin. Aquat. Toxicol. 66 (2), 159–170, Available from http://www.scopus.com/inward/record.url?eid=2-s2.0-1242341224partnerID=40md5=c71a4dc0eea4dbbc082d43c45bc02d03.

Lefebvre, K.A., Bill, B.D., Erickson, A., Baugh, K.A., O'Rourke, L., Costa, P.R., Nance, S., Trainer, V.L., 2008. Characterization of intracellular and extracellular saxitoxin levels in both field and cultured Alexandrium spp samples from Sequim Bay, Washington. Mar. Drugs 6 (2), 103–116, http://dx.doi.org/10.3390/md20080006, Available from http://www.ncbi.nlm.nih.gov/pubmed/18728762.

Liu, Y., Chen, W., Li, D., Shen, Y., Li, G., Liu, Y., 2006a. First report of aphantoxins in China—waterblooms of toxigenic Aphanizomenon flos-aquae in Lake Dianchi. Ecotoxicol. Environ. Saf. 65 (1), 84–92, http://dx.doi.org/10.1016/j.ecoenv.2005.06.012, Available from http://www.sciencedirect.com/science/article/pii/S0147651305001600.

Liu, Y., Chen, W., Li, D., Shen, Y., Liu, Y., Song, L., 2006b. Analysis of paralytic shellfish toxins in aphanizomenon DC-1 from lake Dianchi, China. Environ. Toxicol. 21 (3), 289–295, http://dx.doi.org/10.1002/tox.20182, Available from.

Llewellyn, L.E., Bell, P.M., Moczydlowski, E.G., 1997. Phylogenetic survey of soluble saxitoxin-Binding activity in pursuit of the function and molecular evolution of saxiphilin, a relative of transferrin. Proc.: Biol. Sci. 264, 891–902, Available from http://www.jstor.org/stable/50753 10.2307/50753.

Llewellyn, L.E., Dodd, M.J., Robertson, A., Ericson, G., de Koning, C., Negri, A.P., 2002. Post-mortem analysis of samples from a human victim of a fatal poisoning caused by the xanthid crab, Zosimus aeneus. Toxicon 40 (10), 1463–1469, http://dx.doi.org/10.1016/S0041-0101(02)00164-2, Available from http://www.sciencedirect.com/science/article/pii/S0041010102001642.

Llewellyn, L.E., 2006a. The behavior of mixtures of paralytic shellfish toxins in competitive binding assays. Chem. Res. Toxicol. 19 (5), 661–667, Available from http://dx.doi.org/10.1021/tx050277i.

Llewellyn, L.E., 2006b. Saxitoxin, a toxic marine natural product that targets a multitude of receptors. Nat. Prod. Rep. 23 (2), 200–222, http://dx.doi.org/10.1039/b501296c, Available from http://www.ncbi.nlm.nih.gov/pubmed/16572228.

Long, R.R., Sargent, J.C., Hammer, K., 1990. Paralytic shellfish poisoning: a case report and serial electrophysiologic observations. Neurology 40 (8), 1310–1312, Available from https://www.scopus.com/inward/record.url?eid=2-s2.0-0025105796partnerID=40md5=de9dd781fa885ac3f6c0bfc8ac98b5f7.

Mahmood, N.A., Carmichael, W.W., 1986. Paralytic shellfish poisons produced by the freshwater cyanobacterium Aphanizomenon flos-aquae NH-5. Toxicon 24 (2), 175–186, http://dx.doi.org/10.1016/0041-0101(86)90120-0, Available from http://www.sciencedirect.com/science/article/pii/0041010186901200.

Markensten, H., Moore, K., Persson, I., 2010. Simulated lake phytoplankton composition shifts toward cyanobacteria dominance in a future warmer climate. Ecol. Appl. 20 (3), 752–767, Available from http://www.ncbi.nlm.nih.gov/pubmed/20437961.

McBarron, E.J., Walker, R.I., Gardner, I., Walker, K.H., 1975. Toxicity to livestock of the blue-Green alga anabaena circinalis. Aust. Vet. J. 51 (12), 587–588, Available from 10.1111/j.1751-0813.1975.tb09400.x.

Mihali, T.K., Kellmann, R., Neilan, B.A., 2009. Characterisation of the paralytic shellfish toxin biosynthesis gene clusters in Anabaena circinalis AWQC131C and Aphanizomenon sp NH-5. BMC Biochem. 10 (8), http://dx.doi.org/10.1186/1471-2091-10-81471-2091-10-8, Available from http://www.ncbi.nlm.nih.gov/pubmed/19331657.

Munday, R., Reeve, J., 2013. Risk assessment of shellfish toxins. Toxins (Basel) 5 (11), 2109–2137, http://dx.doi.org/10.3390/toxins5112109, Available from http://www.ncbi.nlm.nih.gov/pubmed/24226039.

Murray, S.A., Mihali, T.K., Neilan, B.A., 2011. Extraordinary conservation, gene loss, and positive selection in the evolution of an ancient neurotoxin. Mol. Biol. Evol. 28 (3), 1173–1182, http://dx.doi.org/10.1093/molbev/msq295, Available from Go to ISI://000287745200005.

Negri, A.P., Jones, G.J., 1995. Bioaccumulation of paralytic shellfish poisoning (PSP) toxins from the cyanobacterium Anabaena circinalis by the freshwater mussel Alathyria condola. Toxicon 33 (5), 667–678, Available from http://www.ncbi.nlm.nih.gov/pubmed/7660371 004101019400180G [pii].

Negri, A.P., Jones, G.J., Hindmarsh, M., 1995. Sheep mortality associated with paralytic shellfish poisons from the cyanobacterium Anabaena circinalis. Toxicon 33 (10), 1321–1329, http://dx.doi.org/10.1016/0041-0101(95)00068-w, Available from http://www.sciencedirect.com/science/article/B6TCS-3YKM5G1-1X/2/52bf776f3b6cc19602d12c0a4d1dca97.

Oberemm, A., Becker, J., Codd, G.A., Steinberg, C., 1999. Effects of cyanobacterial toxins and aqueous crude extracts of cyanobacteria on the development of fish and amphibians. Environ. Toxicol. 14 (1), 77–88, Available from http://www.scopus.com/inward/record.url?eid=2-s2.0-0032992309partnerID=40md5=8a27fd2dab186a579b416d114d14111c.

Onodera, H., Satake, M., Oshima, Y., Yasumoto, T., Carmichael, W.W., 1997. New saxitoxin analogues from the freshwater filamentous cyanobacterium Lyngbya wollei. Nat. Toxins 5 (4), 146–151, http://dx.doi.org/10.1002/19970504NT4, Available from http://dx.doi.org/10.1002/19970504NT4.

Orr, P.T., Jones, G.J., Hamilton, G.R., 2004. Removal of saxitoxins from drinking water by granular activated carbon, ozone and hydrogen peroxide-implications for compliance with the Australian drinking water guidelines. Water Res. 38 (20), 4455–4461, http://dx.doi.org/10.1016/j.watres.2004.08.024, Available from http://www.ncbi.nlm.nih.gov/ubmed/15556220 S0043-1354(04)00421-X [pii].

Oshima, Y., Hasegawa, M., Yasumoto, T., Hallegraeff, G., Blackburn, S., 1987. Dinoflagellate Gymnodinium catenatum as the source of paralytic shellfish toxins in Tasmanian shellfish. Toxicon 25 (10), 1105–1111, http://dx.doi.org/10.1016/0041-0101(87)90267-4, Available from http://www.sciencedirect.com/science/article/pii/0041010187902674.

Paerl, H.W., Paul, V.J., 2012. Climate change: links to global expansion of harmful cyanobacteria. Water Res. 46 (5), 1349–1363, http://dx.doi.org/10.1016/j.watres.2011.08.002, Available from http://www.ncbi.nlm.nih.gov/pubmed/21893330 S0043-1354(11)00438-6 [pii].

Papageorgiou, J., Linke, T.A., Kapralos, C., Nicholson, B.C., Steffensen, D.A., 2004. Extraction of cyanobacterial endotoxin. Environ. Toxicol. 19 (1), 82–87, http://dx.doi.org/10.1002/tox.10152, Available from 10.1002/tox.10152.

Patino, G.A., Isom, L.L., 2010. Electrophysiology and beyond: multiple roles of Na+ channel beta subunits in development and disease. Neurosci. Lett. 486 (2), 53–59, http://dx.doi.org/10.1016/j.neulet.2010.06.050, Available from http://www.ncbi.nlm.nih.gov/pubmed/20600605 S0304-3940(10)00810-4 [pii].

Pearson, L., Mihali, T., Moffitt, M., Kellmann, R., Neilan, B., 2010. On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. Mar. Drugs 8 (5), 1650–1680, Available from http://www.scopus.com/inward/record.url?eid=2-s2.0-77952961827partnerID=40md5=d62f8601ae6177922eac8de3842ba4c4.

Pereira, P., Onodera, H., Do, Andrinolo, Franca, S., Araújo, F., Lagos, N., Oshima, Y., 2000. Paralytic shellfish toxins in the freshwater cyanobacterium Aphanizomenon flos-aquae, isolated from Montargil reservoir. Portugal. Toxicon. 38 (12), 1689–1702, http://dx.doi.org/10.1016/S0041-0101(00)00100-8 (Available from http://www.sciencedirect.com/science/article/pii/S0041010100001008).

Pereira, P., Li, R., Carmichael, W.W., Dias, E., Franca, S., 2004. Taxonomy and production of paralytic shellfish toxins by the freshwater cyanobacterium Aphanizomenon gracile LMECYA40. Eur. J. Phycol. 39 (4), 361–368, http://dx.doi.org/10.1080/09670260410001714723, Available from http://proxy.library.adelaide.edu.au/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=a9h&AN=15243430&site=ehost-live&scope=site.

Pomati, F., Sacchi, S., Rossetti, C., Giovannardi, S., Onodera, H., Oshima, Y., Neilan, B.A., 2000. The freshwater cyanobacterium planktothrix sp. FP1: molecular identification and detection of paralytic shellfish poisoning toxins. J. Phycol. 36 (3), 553–562, Available from http://dx.doi.org/10.1046/j.1529-8817.2000.99181.x.

Pomati, F., Burns, B.P., Neilan, B.A., 2004a. Identification of an Na(+)-dependent transporter associated with saxitoxin-producing strains of the cyanobacterium Anabaena circinalis. Appl. Environ. Microbiol. 70 (8), 4711–4719, http://dx.doi.org/10.1128/AEM.70.8.4711-4719.2004, Available from http://www.ncbi.nlm.nih.gov/pubmed/15294806 70/8/4711 [pii].

Pomati, F., Rossetti, C., Manarolla, G., Burns, B.P., Neilan, B.A., 2004b. Interactions between intracellular Na+ levels and saxitoxin production in Cylindrospermopsis raciborskii T3. Microbiol 150 (2), 455–461, Available from http://www.scopus.com/inward/record.url?eid=2-s2.0-1242318693&partnerID=40&md5=2730458696c684bf1ecbcbe5a40eab3c.

Price, D.W., Kizer, K.W., Hansgen, K.H., 1991. California's paralytic shellfish poisoning prevention program 1927-89. J. Shellfish Res. 10 (1), 119–146, Available from <Go to ISI>://BCI:BCI199293094508.

Ramos, P.B., Diehl, F., dos Santos, J.M., Monserrat, J.M., Yunes, J.S., 2014. Oxidative stress in rats induced by consumption of saxitoxin contaminated drink water. Harmful Algae 37 (0), 68–74, http://dx.doi.org/10.1016/j.hal.2014.04.002 (Available from http://www.sciencedirect.com/science/article/pii/S1568988314000493.

Reichwaldt, E.S., Ghadouani, A., 2012. Effects of rainfall patterns on toxic cyanobacterial blooms in a changing climate: between simplistic scenarios and complex dynamics. Water Res. 46 (5), 1372–1393, http://dx.doi.org/10.1016/j.watres.2011.11.052, Available from http://www.ncbi.nlm.nih.gov/pubmed/22169160 S0043-1354(11)00744-5 [pii].

Reyero, M., Cacho, E., Martínez, A., Vázquez, J., Marina, A., Fraga, S., Franco, J.M., 1999. Evidence of saxitoxin derivatives as causative agents in the 1997 mass mortality of monk seals in the Cape Blanc Peninsula. Nat. Toxins 7 (6),

311–315, Available from 10.1002/1522-7189(199911/12)7:6<311:AID-NT75>3.0.CO;2-I.
Rodrigue, D.C., Etzel, R.A., Hall, S., de Porras, E., Velasquez, O.H., Tauxe, R.V., Kilbourne, E.M., Blake, P.A., 1990. Lethal paralytic shellfish poisoning in Guatemala. Am. J. Trop. Med. Hyg. 42 (3), 267–271, Available from http://www.ncbi.nlm.nih.gov/pubmed/2316796.
Rosati, B., Marchetti, P., Crociani, O., Lecchi, M., Lupi, R., Arcangeli, A., Olivotto, M., Wanke, E., 2000. Glucose- and arginine-induced insulin secretion by human pancreatic Œ ≤ −cells: the role of HERG K+ channels in firing and release. FASEB J. 14 (15), 2601–2610, http://dx.doi.org/10.1096/fj.00-0077com, Available from htt://www.fasebj.org/content/14/15/2601.abstract.
Sanguinetti, M.C., Jiang, C., Curran, M.E., Keating, M.T., 1995. A mechanistic link between an inherited and an acquird cardiac arrthytmia: HERG encodes the IKr potassium channel. Cell 81 (2), 299–307, http://dx.doi.org/10.1016/0092-8674(95)90340-2, Available from http://www.sciencedirect.com/science/article/pii/0092867495903402.
Sato, C., Ueno, Y., Asai, K., Takahashi, K., Sato, M., Engel, A., Fujiyoshi, Y., 2001. The voltage-sensitive sodium channel is a bell-shaped molecule with several cavities. Nature 409 (6823), 1047–1051, Available from http://dx.doi.org/10.1038/35059098.
Savio-Galimberti, E., Gollob, M.H., Darbar, D., 2012. Voltage-gated sodium channels: biophysics, pharmacology, and related channelopathies. Front. Pharmacol. 3, 124, http://dx.doi.org/10.3389/fphar.2012.00124, Available from http://www.ncbi.nlm.nih.gov/pubmed/22798951.
Schönherr, R., Rosati, B., Hehl, S., Rao, V.G., Arcangeli, A., Olivotto, M., Heinemann, S.H., Wanke, E., 1999. Functional role of the slow activation property of ERG K+ channels. Eur. J. Neurosci. 11 (3), 753–760, Available from http://proxy.library.adelaide.edu.au/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=pbh&AN=5185887&site=ehost-live&scope=site.
Schantz, E.J., Mold, J.D., Stanger, D.W., Shavel, J., Riel, F.J., Bowden, J.P., Lynch, J.M., Wyler, R.S., Riegel, B., Sommer, H., 1957. Paralytic shellfish poison. VI. a procedure for the isolation and purification of the poison from toxic clam and mussel tissues. J. Am. Chem. Soc. 79 (19), 5230–5235, Available from http://dx.doi.org/10.1021/ja01576a044.
Schantz, E.J., Ghazarossian, V.E., Schnoes, H.K., Strong, F.M., Springer, J.P., Pezzanite, J.O., Clardy, J., 1975. Letter: the structure of saxitoxin. J. Am. Chem. Soc. 97 (5), 1238, Available from http://www.ncbi.nlm.nih.gov/pubmed/1133383.
Shafer, T.J., Meyer, D.A., Crofton, K.M., 2005. Developmental neurotoxicity of pyrethroid insecticides: critical review and future research needs. Environ. Health Perspect. 113 (2), 123–136, http://dx.doi.org/10.1289/ehp.7254, Available from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1277854/.
Shimizu, Y., 1993. Microalgal metabolites. Chem. Rev. 93 (5), 1685–1698, Available from http://dx.doi.org/10.1021/cr00021a002.
Shinohara, R., Akimoto, T., Iwamoto, O., Hirokawa, T., Yotsu-Yamashita, M., Yamaoka, K., Nagasawa, K., 2011. Synthesis of skeletal analogues of saxitoxin derivatives and evaluation of their inhibitory activity on sodium ion channels NaV1.4 and NaV1.5. Chem. Eur. J. 17 (43), 12144–12152, Available from http://dx.doi.org/10.1002/chem.201101058.
Song, C., Kanthasamy, A., Kanthasamy, A., 2011. Chapter 63 – Cell signaling mechanisms in developmental neurotoxicity. In: Reproductive and Developmental Toxicology. Academic Press, San Diego, pp. 835–845.
Stevens, M., Peigneur, S., Tytgat, J., 2011. Neurotoxins and their binding areas on voltage-gated sodium channels. Front. Pharmacol. 2, 71, http://dx.doi.org/10.3389/fphar.2011.00071, Available from http://www.ncbi.nlm.nih.gov/pubmed/22084632.
Stewart, I., Webb, P.M., Schluter, P.J., Fleming, L.E., Burns Jr., J.W., Gantar, M., Backer, L.C., Shaw, G.R., 2006. Epidemiology of recreational exposure to freshwater cyanobacteria–an international prospective cohort study. BMC Public Health 6, 93, http://dx.doi.org/10.1186/1471-2458-6-93, Available from http://www.ncbi.nlm.nih.gov/pubmed/16606468.
Strassburg, C.P., Strassburg, A., Kneip, S., Barut, A., Tukey, R.H., Rodeck, B., Manns, M.P., 2002. Developmental aspects of human hepatic drug glucuronidation in young children and adults. Gut 50 (2), 259–265, Available from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1773113/.
Su, Z., Sheets, M., Ishida, H., Li, F., Barry, W.H., 2004. Saxitoxin blocks L-type ICa. J. Pharmacol. Exp. Ther. 308 (1), 324–329, http://dx.doi.org/10.1124/jpet.103.056564, Available from http://www.ncbi.nlm.nih.gov/pubmed/14566004.
Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T., Numa, S., 1987. Primary structure of the receptor for calcium channel blockers from skeletal muscle. Nature 328 (6128), 313–318, Available from http://dx.doi.org/10.1038/328313a0.
Tanaka, J., Toku, K., Zhang, B., Ishihara, K., Sakanaka, M., Maeda, N., 1999. Astrocytes prevent neuronal death induced by reactive oxygen and nitrogen species. Glia 28 (2), 85–96, Available from 10.1002/(SICI)1098-1136(199911)28:2<85:AID-GLIA1>3.0.CO;2-Y.
Terlau, H., Heinemann, S.H., Stühmer, W., Pusch, M., Conti, F., Imoto, K., Numa, S., 1991. Mapping the site of block by tetrodotoxin and saxitoxin of sodium channel II. FEBS Lett. 293 (1,2), 93–96, http://dx.doi.org/10.1016/0014-5793(91)81159-6, Available from http://www.sciencedirect.com/science/article/pii/0014579391811596.
Torres, R., Pizarro, L., Csendes, A., Garc Iacute, A.C., Lagos, N., eacute stor, 2007. GTX 2/3 epimers permeate the intestine through a paracellular pathway. J. Toxicol. Sci. 32 (3), 241–248 (Available).
Van Dolah, F.M., 2000. Marine algal toxins: origins, health effects, and their increased occurrence. Environ. Health Perspect. 108 (suppl 1), 133–141, Available from http://www.ncbi.nlm.nih.gov/ubmed/10698729 sc271_5_1835 [pii].
Wacklin, P., Hoffman, L., Komárek, J., 2009. Nomenclatural validation of the genetically revised cyanobacterial genusDolichospermum(RALFS ex BORNET et FLAHAULT) comb. nova. Fottea 9 (1), 59–64, Available.
Walker, J.R., Novick, P.A., Parsons, W.H., McGregor, M., Zablocki, J., Pande, V.S., Du Bois, J., 2012. Marked difference in saxitoxin and tetrodotoxin affinity for the human nociceptive voltage-gated sodium channel (Nav1.7). Proc. Natl. Acad. Sci. 109 (44), 18102–18107, http://dx.doi.org/10.1073/pnas.1206952109, Available from http://www.nas.org/content/109/44/18102.abstract.
Wang, J., Salata, J.J., Bennett, P.B., 2003. Saxitoxin is a gating modifier of HERG K+ channels. J. Gen. Physiol. 121 (6), 583–598, http://dx.doi.org/10.1085/jgp.200308812jgp.200308812, Available from http://www.ncbi.nlm.nih.gov/pubmed/12771193.
Wekell, J.C., Hurst, J., Lefebvre, K.A., 2004. The origin of the regulatory limits for PSP and ASP toxins in shellfish. J. Shellfish Res. 23 (3), 927–930, Available from https://www.scopus.com/inward/record.url?eid=2-s2.0-13544267579partnerID=40md5=7c7f737be8ee6b37a2d9d4431360adad.
Wiese, M., D'Agostino, P.M., Mihali, T.K., Moffitt, M.C., Neilan, B.A., 2010. Neurotoxic alkaloids: saxitoxin and its analogs. Mar. Drugs 8 (7), 2185–2211, http://dx.doi.org/10.3390/md8072185, Available from http://www.ncbi.nlm.nih.gov/pubmed/20714432.
Yang, I., John, U., Beszteri, S., Glockner, G., Krock, B., Goesmann, A., Cembella, A.D., 2010. Comparative gene expression in toxic versus non-toxic strains of the marine dinoflagellate Alexandrium minutum. BMC Genom. 11, 248, http://dx.doi.org/10.1186/1471-2164-11-2481471-2164-11-248, Available from http://www.ncbi.nlm.nih.gov/pubmed/20403159.
Yotsu-Yamashita, M., Sugimoto, A., Terakawa, T., Shoji, Y., Miyazawa, T., Yasumoto, T., 2001. Purification, characterization, and cDNA cloning of a novel soluble saxitoxin and tetrodotoxin binding protein from plasma of the puffer fish, Fugu pardalis. Eur. J. Biochem. 268 (22), 5937–5946, Available from http://dx.doi.org/10.1046/j.0014-2956.2001.02547.x.
Zegura, B., Straser, A., Filipic, M., 2011. Genotoxicity and potential carcinogenicity of cyanobacterial toxins – a review. Mutat. Res. 727 (1-2), 16–41, http://dx.doi.org/10.1016/j.mrrev.2011.01.002, Available from http://www.ncbi.nlm.nih.gov/pubmed/21277993 S1383-5742(11)00003-2 [pii].

Appendix 2

O'Neill, K., Musgrave, I.F. and Humpage, A. (2017). Extended low-dose exposure to saxitoxin inhibits meurite outgrowth in model neuronal cells. *Basic and Clinical Pharmacology and Toxicology,* 120(4), 390-397.