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Comparison of the acute inhibitory effects of Tetrodotoxin (TTX) in rat and human neuronal networks for risk assessment purposes

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HIGHLIGHTS

- TTX inhibits neuronal electrical activity in rat cortical cultures (IC_{50} ~7 nM).
- TTX is equipotent in human iPSC-derived neurons (IC_{50} ~10 nM).
- Our and literature data indicate that interspecies differences for TTX are limited.
- Experimental animal data could be used to derive human acute reference dose (ARfD).
- The ARfD amounts to 1.33 μg/kg bw, or 200 μg/kg TTX in shellfish.

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ABSTRACT

Tetrodotoxin (TTX) is an extremely toxic marine neurotoxin. TTX inhibits voltage-gated sodium channels, resulting in a potentially lethal inhibition of neurotransmission. Despite numerous intoxications in Asia and Europe, limited (human) toxicological data are available for TTX. Additionally, the degree of interspecies differences for TTX is not well established, hampering the use of available (animal) data for human risk assessment and establishing regulatory limits for TTX concentrations in (shell)fish.

We therefore used micro-electrode array (MEA) recordings as an integrated measure of neurotransmission to demonstrate that TTX inhibits neuronal electrical activity in both primary rat cortical cultures and human-induced pluripotent stem cell (hIPSC)-derived iCell® neurons in co-culture with hIPSC-derived iCell® astrocytes, with IC_{50} values of 7 and 10 nM, respectively.

From these data combined with LD_{50} values and IC_{50} concentrations of voltage-gated sodium channels derived from literature it can be concluded that interspecies differences are limited for TTX. Consequently, we used experimental animal data to derive a human acute reference dose of 1.33 μg/kg body weight, which corresponds to maximum concentration of TTX in shellfish of 200 μg/kg.

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1. Introduction

Tetrodotoxin (TTX) is a potent neurotoxin that is naturally present in certain marine and terrestrial species. The *fugu* or puffer fish (*Tetraodontidae* family) is the best known source of TTX (Bane et al., 2014; Noguchi and Arakawa, 2008). TTX is not produced by puffer fish itself, but most likely originates from a symbiosis of bacteria with marine animals (Lago et al., 2015). TTX is a voltage-gated sodium channel blocker, which binds to the sodium channel and thereby prevents the flux of sodium ions through the channel. Consequently, depolarization of the nerve cell membrane and generation of action potential is prevented, resulting in inhibition of neurotransmission in the central and peripheral nervous system (Bane et al., 2014). In total, 26 naturally occurring analogues of TTX are known, which may differ in potency but have comparable toxicological properties (Bane et al., 2014, 2016; Miyazawa and Noguchi, 2001). Intoxicated patients suffer from paresthesia of the

Abbreviations: ARfD, acute reference dose; DIV, days *in vitro*; EFSA, European Food Safety Authority; FBS, fetal bovine serum; hIPSC, human-induced pluripotent stem cell; IC_{50}, half maximal inhibitory concentration; LD_{50}, median lethal dose; LOEAL, lowest observed adverse effect level; (mw)MEA, (multi-well)micro-electrode array; MLD, minimum lethal dose; MSR, mean spike rate; NBA, Neurobasal®-A; NOAEL, no observed adverse effects levels; PEI, polyethyleneimine; Pen/Strep, penicillin/streptomycin; PND, post-natal day; TTX, tetrodotoxin.

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tongue and lips, headache, vomiting, ataxia and in severe cases respiratory- and heart failure (Bane et al., 2014; Lago et al., 2015). While the presence of TTX used to be confined to Asia and especially Japan, TTX has also been found in the last decade in marine organisms, including shellfish, in Europe (Turner et al., 2015; Rodriguez et al., 2008; Vlamis et al., 2015). As such, the appearance of TTX in European shellfish is an emerging problem and uniform regulatory limits for TTX have not yet been set as TTX is currently under review by the European Food Safety Authority (EFSA).

Unfortunately, it is hard to establish regulatory limits for TTX since human data is based on case studies. Human no observed adverse effects levels (NOAELs) or lethal human levels are therefore not reliable. There are reports of LD_{50} values for mice and cats, but the extrapolation to the human situation is difficult due to limited knowledge regarding interspecies differences for TTX. It is therefore of great importance to compare animal and human models to determine if interspecies difference in sensitivity to TTX exist.

Since TTX inhibits neurotransmission by blocking voltage-gated sodium channels, measurements of neuronal network activity *in vitro* provide a suitable integrated endpoint to assess effects of TTX on neuronal signaling (Nicolas et al., 2014). Using micro-electrode array (MEA) recordings, changes in (spontaneous) neuronal electrical activity can be measured *in vitro* (Johnstone et al., 2010). Analogues to the *in vivo* situation, neuronal networks grown on MEAs develop spontaneous activity (Robinette et al., 2011; Dingemans et al., 2016; de Groot et al., 2016) and are responsive to different neurotransmitters and chemicals (Hondebrink et al., 2016). While rat primary cortical cultures are currently the golden standard for MEA recordings, the use of human induced pluripotent stem cell (hIPSC)-derived neuronal models is increasing (Tukker et al., 2016), thereby potentially eliminating the need for interspecies extrapolation. The aim of the present study is therefore to compare the sensitivity of rat primary cortical cultures and co-cultures of hIPSC-derived iCell® neurons and hIPSC-derived iCell® astrocytes to the inhibitory effects of TTX on neuronal electrical activity using MEA recordings, and to calculate an acute reference dose (ARfD) for TTX taking into account this interspecies extrapolation.

2. Materials and methods

2.1. Chemicals

Tetrodotoxin citrate (TTX, purity >98%) was obtained from Abcam (Cambridge, United Kingdom). Neurobasal®-A (NBA) Medium, L-glutamine, fetal bovine serum (FBS), B-27 supplement, KnockOut Serum Replacement, 50/50 DMEM/F12 medium and penicillin-streptomycin (Pen/Strep) (10,000 U/mL–10,000 μg/mL) were purchased from Life Technologies (Bleiswijk, The Netherlands). iCell® Neurons Maintenance Medium (NRM-100-121-001) and iCell® Neurons Medium Supplement (NRM-100-031-001) were purchased from Cellular Dynamics International (Madison, WI, USA). All other chemicals were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Stock solutions of TTX (1 mM) were prepared in MilliQ® water and stored at 4 °C for a maximum of 3 months. Stock solutions were diluted in cell culture medium to obtain the desired concentrations just prior to the experiments.

2.2. Cell culture

All cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C.

2.2.1. Rat primary cortical cells

Rat primary cortical cells were isolated from the neonatal cortex from post-natal day (PND) 1 Wistar rat pups as described previously (Dingemans et al., 2016; de Groot et al., 2016; Hondebrink et al., 2016). Briefly, rat pups were decapitated and cortices were rapidly dissected on ice. Tissues were kept in dissection medium containing NBA medium, supplemented with 25 g/L sucrose, 450 μM L-glutamine, 30 μM glutamate, 1% Pen/Strep and 10% FBS, pH was set to 7.4. Cells were seeded in dissection medium on poly-L-lysine (50 μg/mL) coated culture materials. After 1 day in culture (DIV1), 90% of the dissection medium was replaced with comparable medium, but with 2% B-27 supplement instead of FBS. At DIV4, 90% of the medium was replaced with NBA medium, supplemented with 15 g/L sucrose, 450 μM <***>l-glutamine, 1% Pen/Strep and 10% FBS, pH was set to 7.4 (glutamate-free medium). For MEA experiments, a 50 μL drop of cell suspension ($1*10^5$ cells/well) was placed on the electrode field in each well of the 48-wells of a multi-well MEA plate. All animal experiments were performed in accordance with the Dutch law and were approved by the Ethical Committee for Animal Experimentation of Utrecht University (project number AVD108002015443). All efforts were made to treat the animals humanely and for alleviation of suffering.

2.2.2. iCell® neurons/astrocytes

iCell® neurons (NRC-100-010-001, Cellular Dynamics International (CDI), Madison, WI, USA) and iCell® astrocytes (ASC-100-020-001-PT, CDI, Madison, WI, USA) were seeded as an iCell® neurons/iCell® astrocytes co-culture according to CDI protocol with slight modifications. Briefly, iCell® neurons and iCell® astrocytes were seeded as a 10 μL droplet of cell suspension at a density of $1.4*10^5$ cells/μL, with $7.0*10^4$ iCell® neurons and $7.0*10^4$ astrocytes in Complete iCell® Neurons Maintenance Medium, supplemented with 2% iCell® Neurons Medium Supplement and 10 μg/mL laminin. The cell suspension was placed directly over the electrode field of each polyethyleneimine (PEI)-coated well of a 48-well MEA plate (Axion Biosystems Inc., Atlanta, USA) that has been pre-dotted with 10 μL of iCell® Neurons Maintenance medium with 80 μg/mL laminin. The cell suspension droplet was allowed to attach to the electrode field for 35 min, after which 300 μL of Complete iCell® Maintenance Medium supplemented with laminin was added to each well. Every 2–3 days, 50% of the medium was refreshed.

2.3. Multi-well microelectrode array recordings

Multi-well microelectrode array (mwMEA) plates contained 48 wells per plate, with each well containing an electrode array of 16 individual embedded nanotextured gold microelectrodes with four integrated ground electrodes, yielding a total of 768 channels (Axion Biosystems Inc.). Spontaneous electrical activity was recorded as described previously (Dingemans et al., 2016; de Groot et al., 2016; Hondebrink et al., 2016; Tukker et al., 2016). Briefly, experiments were performed at DIV9 (rat cortical cultures) or at DIV7 (iCell® neurons/astrocytes) at 37 °C, using a Maestro 768-channel amplifier with integrated heating system, temperature controller and data acquisition interface (Axion BioSystems Inc., Atlanta, USA).

After a 5 min equilibration period, a 30 min baseline recording of the spontaneous activity was started. Only wells with at least one visibly active electrode after equilibration were included. After the baseline recording, 33 μL TTX (final concentrations 1–30 nM, diluted in NBA medium, supplemented with 10% KnockOut Serum Replacement and 1% Pen/Strep) was added to the wells (iCell® neurons/astrocytes). For rat cortical cultures, 5 μL TTX (diluted in FBS medium) was added to the wells. Following addition of TTX

(final concentrations 1–30 nM) activity was recorded for another 30 min to determine the inhibitory effect of TTX on neuronal activity.

2.4. Data analysis and statistics

Data were analyzed by NeuroExplorer5 software (NEX Technologies, Madison, USA) and custom-made MS Excel macros as described previously (Hondebrink et al., 2016). Raw data files were re-recorded for spike detection using the AxIS spike detector (Adaptive threshold crossing, Ada BandFit v2) with a threshold spike detector set at 7*SD (rat cortical culture) or 6*SD (iCell® neurons/astrocytes co-culture) of the internal noise level on each electrode. The re-recorded raw data were loaded into NeuroExplorer to determine the average mean spike rate (MSR; spikes/s) of all active electrodes per active well. Electrodes were considered active when MSR > 0.1 spikes/s and wells were considered active when ≥1 active electrode was present. The development of spontaneous activity (MSR) over time and the effect of TTX on spontaneous activity was determined by custom-made MS Excel macros. The MSR during the exposure recording ($MSR_{exposure}$) was expressed as a percentage of the MSR during the baseline recording ($MSR_{baseline}$) to derive a treatment ratio. Treatment ratios of individual electrodes were averaged per well and subsequently per condition. Outliers (effects 2*SD above or below average) in control and effect data were excluded for further analysis (<5%). The treatment ratios in control experiments were set to 100% and effects of TTX were expressed as percentage of control. For all data analysis, GraphPad Prism v6.05 (GraphPad Software, San Diego, California) was used. Groups were compared using an ANOVA followed by a post-hoc Bonferroni test. Effects were considered statistically significant when $p < 0.05$.

3. Results

Effects of acute TTX exposure were investigated in rat cortical cultures and iCell® neurons/astrocytes co-cultures. Following a baseline recording (Fig. 1A), cultures were exposed for 30 min to different concentrations of TTX (Fig. 1B). Each well was exposed to only one concentration to prevent potential effects of cumulative dosing.

In rat primary cortical cultures, TTX concentration-dependently inhibits neuronal activity (Fig. 1C). At 3 nM and 10 nM, TTX inhibited neuronal activity significantly to 75% ($p < 0.05$) and 25% ($p < 0.0001$) from control, respectively. At 30 nM, neuronal activity of rat primary cortical cultures was almost completely abolished and the calculated IC_{50} for inhibition of neuronal activity by TTX amounted to 7 nM.

In human iPSC-derived iCell® neurons/astrocytes co-cultures, TTX induced a comparable concentration-dependent inhibition of neuronal activity (Fig. 1C). At 3 nM and 10 nM, TTX inhibited neuronal activity significantly to 78% ($p < 0.0001$) and 41% ($p < 0.0001$) from control, respectively. Comparable with rat primary cortical cultures, 30 nM TTX almost completely abolished neuronal activity and the calculated IC_{50} for inhibition of neuronal activity by TTX amounted to 10 nM.

4. Discussion

We earlier demonstrated the suitability of rat cortical cultures grown on mwMEAs to investigate the effects of various marine neurotoxins, including TTX, on neuronal activity (Nicolas et al., 2014). Here we investigated the acute inhibitory effects of TTX on neuronal electrical activity in rat primary cortical cultures and iCell® neurons/astrocytes co-cultures. It is important to note that currently most *in vitro* models do not sufficiently take into account

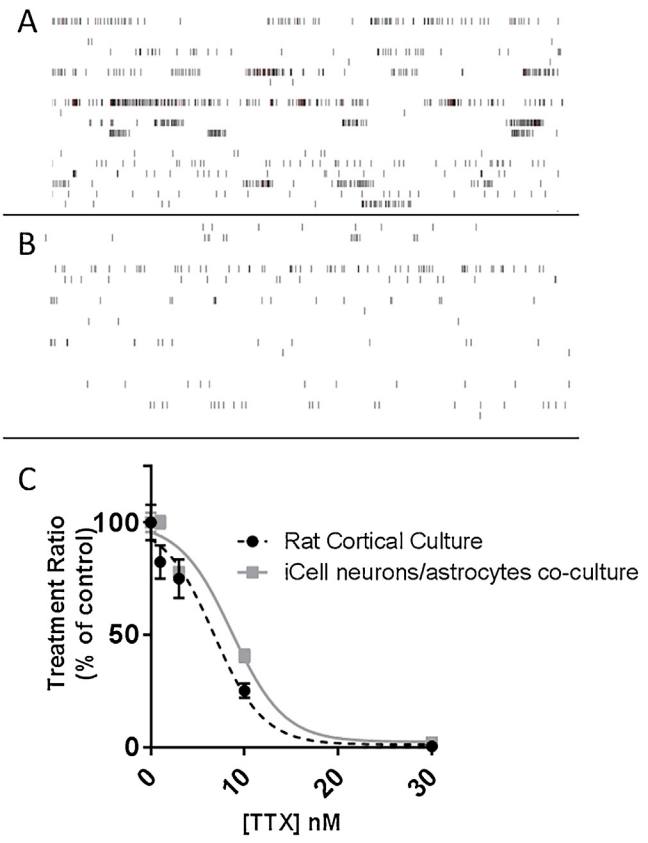

Fig. 1. Examples of Spike Raster Plots of iCell® neurons/astrocytes co-cultures before (A) and after (B) exposure to 10 nM TTX. Each row depicts one electrode; every mark represents one spike. Concentration-effect curves (C) of TTX on neuronal activity in primary rat cortical cultures (black dotted line, $n_{wells} = 22–29$, $N_{plates} = 4$) and iCell® neurons/astrocytes co-cultures (grey solid line, $n_{wells} = 7–24$, $N_{plates} = 1–3$). Results are expressed as treatment ratio ± SEM (change in% between $MSR_{baseline}$ and $MSR_{exposure}$). Data were synchronized to the time of exposure to eliminate the application artifact (Hondebrink et al., 2016). Effects of TTX exposure are significantly different from control at ≥3 nM.

factors such as bioactivation/metabolisms or exposure routes such as the gastrointestinal tract. *In vitro* data, such as our measurements of neuronal electrical activity using MEA recordings, thus cannot (yet) fully replace experimental animal studies for risk assessment purposes. Nevertheless, these *in vitro* studies can shed light on the degree of interspecies variation, which can subsequently be used to further shape risk assessments based on (existing) experimental data.

Our results demonstrate that TTX potently and concentration-dependently inhibits neuronal electrical activity in both models. The IC_{50}s for inhibition of rat cortical cultures obtained in the present study are in line with those obtained earlier [Nicolas et al., 2014, $IC_{50} = 4$ nM], demonstrating the reproducibility of this approach. Interestingly, neuronal activity completely ceased at 30 nM TTX, which is close to the estimated lethal blood level of 9 ng/mL TTX (28 nM) (Bane et al., 2014; Islam et al., 2011; Leung et al., 2011). More importantly, TTX is roughly equipotent in both the rat and human in vitro models, indicating that for inhibition of neuronal activity by TTX, rat-human interspecies differences are limited.

Notably, interspecies differences appear to be also limited for TTX-induced inhibition of voltage-gated sodium channel, which are highly conserved in mammalian species (Goldin et al., 2000; Lopreato et al., 2001; Grosson et al., 1996). For example, TTX is

roughly equipotent in different species in inhibiting voltage-gated $Na_V1.4$ sodium channels from skeletal muscle (rat IC_{50} = 17 nM (Walker et al., 2012), human IC_{50} = 25 nM (Chahine et al., 1994)), voltage-gated $Na_V1.6$ sodium channels from the central nervous system (rat IC_{50} = 1 nM (Dietrich et al., 1998), mouse IC_{50} = 6 nM (Smith et al., 1998)), and voltage-gated $Na_V1.7$ sodium channels from dorsal root ganglia in the peripheral nervous system (rat IC_{50} = 4 nM (Sangameswaran et al., 1997), human IC_{50} = 19–25 nM (Walker et al., 2012; Klugbauer et al., 1995)). Similarly, when comparing minimum lethal dose (MLD) levels in mammals following subcutaneous injection, little interspecies differences are observed with rats and guinea pigs being most sensitive (estimated MLD of crystalline TTX of 2.7 μg/kg and 4.5 μg/kg, respectively) and mouse, dogs and cats being least sensitive (estimated MLD of crystalline TTX of 8 μg/kg, 9 μg/kg and 10 μg/kg, respectively) (Kao, 1966). Since at least for TTX rat-human interspecies differences appear limited, experimental animal (rat) data for TTX may play a more prominent role in human risk assessment of TTX.

Current human risk assessment is hampered by the lack of well-documented human intoxications. While a grading system for the severity of TTX poisoning was already provided in 1941 (Fukuda and Tani, 1941) and numerous reports on TTX intoxication exist, only a few measured urine and/or blood concentrations of patients and none can provide a reliable estimate of the amount of TTX ingested. Human lethal dose estimations of 1–4 mg reported earlier (Zimmer, 2010; Klaassen, 2013) should therefore be treated with caution. It thus seems viable to use experimental animal data instead.

Based on experimental animal data, the oral LD_{50} value of TTX in mice is reported to be 334–700 μg/kg (Cowawintaweewat et al., 2011; Melorose et al., 2005), whereas 200 μg/kg was reported to be a non-lethal dose in cats (Murtha et al., 1958). Since TTX is ~50 times less toxic orally than intraperitoneally/subcutaneously (Xu et al., 2003), these values are in good agreement with the MLD values (Kao, 1966). Given the lack of interspecies differences, 400 μg/kg can be assumed to be an appropriate LD_{50} value for mammals.

Using this 'universal' mammalian LD_{50} value (400 μg/kg) and appropriate safety factors, an acute reference dose (ARfD) for TTX can be estimated that is likely to be more reliable than those based on human case studies. Given the steepness of for example the concentration-effect curve for inhibition of neuronal activity (Fig. 1C) and its close correlation to estimated human lethal blood levels (Bane et al., 2014; Islam et al., 2011; Leung et al., 2011), a conservative factor 10 can be used to get from a LD_{50} value to a lowest observed adverse effect level (LOAEL) value (40 μg/kg). A subsequent factor 3 can be applied to get from LOAEL to a no observed adverse effect level (NOAEL) value, yielding a conservative NOAEL of 13.33 μg/kg for TTX in humans.

To account for human risk groups, an additional safety factor has to be applied. Since TTX intoxications are reported in all different age groups (Islam et al., 2011; Kanchanapongkul, 2008; Azizul Haque et al., 2008), it appears all age groups are prone to TTX poisoning (Bane et al., 2014). While naturally occurring mutations and polymorphisms in voltage-gated sodium channels do occur, no studies were found in literature that compared wild-type and mutant channels for TTX affinity or demonstrated an increased TTX sensitivity in people with a polymorphism for one of the sodium channels. Nevertheless, human risk groups likely exist on a pharmacokinetic level.

After ingestion, TTX serum concentrations fall rapidly, whereas TTX appears rapidly and disappears slowly from the kidneys suggesting it is mainly excreted via urine (Kao, 1966; O'Leary et al., 2004). Although hard evidence is lacking, it can therefore be assumed that people with reduced kidney function and/or poor hydration status could be more prone to TTX poisoning, due to delayed excretion of TTX. This is in agreement with the suggestion that people who suffer from diabetic neuropathy, uremia and Na-K-adenosine-triphosphate deficiency can have more severe TTX-induced adverse effects than healthy patients (Bane et al., 2014; Leung et al., 2011). Consequently, a subsequent factor 10 should be applied to take into account any intraspecies differences due to for example poor renal clearance and/or poor hydration status. This would result in an ARfD for TTX of 1.33 μg/kg bw. Using this ARfD and assuming "400 g of shellfish meat as an appropriate estimate of a large portion size consumed in Europe to be used in its risk assessments in order to protect high consumers against acute effects of marine biotoxins" (EFSA, 2010), a person of 60 kg can safely consume shellfish with a maximum concentration of TTX of 200 μg/kg.

Conflict of interest

The authors state that they have no conflict of interest.

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