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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.

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