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Mixture effects of tetrodotoxin (TTX) and drugs targeting voltage-gated sodium channels on spontaneous neuronal activity *in vitro*

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ABSTRACT

Tetrodotoxin (TTX) potently inhibits TTX-sensitive voltage-gated sodium (Na_V) channels in nerve and muscle cells, potentially resulting in depressed neurotransmission, paralysis and death from respiratory failure. Since a wide range of pharmaceutical drugs is known to also act on Na_V channels, the use of medicines could predispose individuals to a higher susceptibility towards TTX toxicity. We therefore first assessed the inhibitory effect of selected medicines that act on TTX-sensitive (Riluzole, Chloroquine, Fluoxetine, Valproic acid, Lamotrigine, Lidocaine) and TTX-resistant (Carbamazepine, Mexiletine, Flecainide) Na_V channels on spontaneous neuronal activity of rat primary cortical cultures grown on microelectrode arrays (MEA). After establishing concentration-effect curves, binary mixtures of the medicines with TTX at calculated NOEC, IC_{20} and IC_{50} values were used to determine if pharmacodynamic interactions occur between TTX and these drugs on spontaneous neuronal activity. At IC_{20} and IC_{50} values, all medicines significantly increased the inhibitory effect of TTX on spontaneous neuronal activity of rat cortical cells *in vitro*. Subsequent experiments using human iPSC-derived neuronal co-cultures grown on MEAs confirmed the ability of selected medicines (Carbamazepine, Flecainide, Riluzole, Lidocaine) to inhibit spontaneous neuronal activity. Despite the need for additional experiments using human iPSC-derived neuronal co-cultures, our combined data already highlight the importance of identifying and including vulnerable risk groups in the risk assessment of TTX.

1. Introduction

Tetrodotoxin (TTX) is an extremely potent neurotoxin that is naturally occurring in certain marine species and has long been known to be responsible for human intoxications, especially in Asia (Lago et al., 2015). The most well-known source of TTX is the puffer fish, though it is not produced by the fish itself but by certain symbiotic bacteria. Other seafood species potentially carrying TTX include gastropods, starfish, crabs, octopuses, goby fishes, frogs, and newts (Biessy et al., 2019). Since 2015, TTX has frequently been detected in various marine species in Europe. As such, TTX has been detected in mussels, oysters and clams in the UK, Greece and the Netherlands, in quantities ranging from 20 μg/kg to 253 μg/kg (Turner et al., 2015; Rodriguez et al., 2008; Vlamis et al., 2015). Exposure to such amounts of TTX after consumption of marine species could possibly result in serious adverse health effects. Since uniform regulatory limits have not yet been determined in the EU, the emergence of TTX is becoming an increasing concern.

TTX selectively binds to voltage-gated sodium (Na_V) channels in nerve and muscle cells, thereby blocking the influx of sodium ions through the channel and consequently the generation and propagation of action potentials (Lee and Ruben, 2008). This will result in depressed neurotransmission within the (peripheral) nervous system and paralysis of nerve and muscle function, which will eventually lead to diaphragm paralysis and death from respiratory failure. Up to date, nine functional

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mammalian isoforms of Na_V channels have been identified ($Na_V1.1$--$Na_V1.9$) (Bagal et al., 2015). Distribution of these isoforms varies from the central nervous system ($Na_V1.1$-$Na_V1.3$ and $Na_V1.6$), to the peripheral nervous system ($Na_V1.7$-$Na_V1.9$), skeletal muscle ($Na_V1.4$) and the heart ($Na_V1.5$). TTX is known to selectively block $Na_V1.1$, $Na_V1.2$, $Na_V1.3$ and $Na_V1.7$ (i.e. the TTX-sensitive Na_V channels) in the skeletal muscles and the central nervous system. The other Na_V channels are generally insensitive to TTX binding and therefore considered as TTX-resistant Na_V channels.

Although sufficient data is available on the sensitivity of different animal species for TTX intoxications (Melorose et al., 2005; Murtha, 1958; Xu et al., 2003; Kao, 1966), there is a general lack of reliable dose-response data on human sensitivity to the effects of TTX. This complicates the current risk assessment process and especially the establishment of reliable human 'no observed adverse effects levels' (NOAELs). The use of alternative animal or *in vitro* models with proven minimal interspecies differences is therefore becoming increasingly important.

We have previously shown that TTX is extremely potent and concentration-dependently inhibits neuronal activity in rat primary cortical cultures grown on multi-well microelectrode arrays (MEA), with an IC_{50} value of ~4 nM (Nicolas et al., 2014). Notably, comparably low IC_{50} values (~7 nM) were determined in a subsequent study, highlighting the reproducibility of MEA recordings (Kasteel and Westerink, 2017). Moreover, minimal interspecies differences were found between rat and human neurons (IC_{50} value of ~10 nM), and these models can therefore be considered viable alternatives for human data in the risk assessment of TTX. Importantly, the concentration-effect curve for inhibition of neuronal activity by TTX is extremely steep, with full inhibition already at 30 nM in both rat and human neuronal cultures (Nicolas et al., 2014).

The identification of human risk groups constitutes an important step in the risk assessment process. In general, all age groups are reported to be prone to TTX poisoning and there is currently no evidence available that highlights specific sensitive groups (Bane et al., 2014). However, the use of medicines could predispose individuals to a higher susceptibility towards TTX toxicity, particularly by pharmacodynamic or pharmacokinetic interactions. Since Na_V channels play an essential role in the initiation and propagation of action potentials in excitable neuronal cells, muscles, and cardiac tissue, these channels have been clinically targeted by a wide range of pharmaceutical drugs for a diverse range of conditions including chronic pain, epilepsy, and cardiac arrhythmias (Bagal et al., 2015; Waszkielewicz et al., 2013). Examples of drug classes that act on either TTX-sensitive or TTX-resistant Na_V channels include anticonvulsants (e.g., Valproic acid, Lamotrigine, Carbamazepine), antiarrhythmics (e.g., Mexiletine, Flecainide), neuroprotective agents (e.g. Riluzole), anti-viral drugs (e.g., Chloroquine), anti-depressants (e.g., Fluoxetine), and local anaesthetics (e.g., Lidocaine) that are administered to successfully control Na^+ influx through Na_V channels. However, to what extent these drugs exhibit inhibiting properties on spontaneous neuronal network activity in rat and human neuronal co-cultures has not been investigated yet.

Importantly, it is unknown if patients using the abovementioned medicines have an increased risk for the toxic effects of TTX. Non-toxic doses of TTX could become toxic in combination with these medicines due to additive or synergistic interactions. Until now, such interactions between TTX and medicines have not been investigated. Therefore, the current study aims to determine the inhibitory effects of a selection of medicines that target Na_V channels on spontaneous neuronal activity using rat primary cortical cultures and hiPSC-derived neuronal co-cultures. In addition, the potential pharmacodynamic interaction of these medicines with TTX was investigated. Our data show that the selected medicines decreased spontaneous neuronal activity of both rat and human neuronal co-cultures in a concentration-dependent manner. Moreover, significant augmentation of the TTX-induced inhibition of spontaneous neuronal activity of rat primary cortical neurons is observed when neurons were exposed to binary mixtures of TTX with medicines.

2. Materials and methods

2.1. Chemicals

Neurobasal®-A (NBA) Medium, L-glutamine, foetal bovine serum (FBS), penicillin-streptomycin (Pen/Strep) (10,000 U/mL-10,000 μg/mL) and B27 supplement (without vitamin A) were purchased from Life Technologies (Bleiswijk, The Netherlands). SynFire® glutamatergic neurons, SynFire® GABAergic neurons, SynFire® astrocytes, neuronal seeding medium, short-term maintenance medium and long-term maintenance medium were purchased from NeuCyte (Sunnyvalle, CA, USA). Tetrodotoxin citrate (TTX, purity >98 %) was purchased from Alomone Labs (Jerusalem, Israel). Chloroquine, Fluoxetine, Riluzole, 50 % polyethyleneimine (PEI), sodium borate, boric acid solution, laminin (L2020) and all other chemicals were purchased 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 of Chloroquine, Carbamazepine, Lamotrigine, Mexiletine, Flecainide, Fluoxetine and Riluzole were prepared in dimethyl sulfoxide (DMSO) in dilution series and stored at 4°C. Stock solutions of Valproic acid and Lidocaine were freshly prepared before every experiment in FBS culture medium (rat cortical cultures) or long-term maintenance medium (hiPSC-derived neuronal co-cultures). 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. All cell culture surface materials were pre-coated with 0.1 % PEI solution diluted in borate buffer (24 mM sodium borate / 50 mM boric acid in Milli-Q, pH adjusted to 8.4). In the case of SynFire® neurons, PEI coating was followed by a laminin coating for which the electrode grids were covered with a 70 μL droplet of 20 μg/mL laminin in PBS for one hour at 37 °C. This coating was removed directly before plating the cells.

2.2.1. Rat primary cortical cells

Timed-pregnant (E18) Wister rats were obtained from Envigo Laboratories B.V. (Horst, The Netherlands). All animal experiments were performed in accordance with Dutch law and were approved by the Ethical Committee for Animal Experimentation of Utrecht University. All efforts were made to treat the animals humanely and for alleviation of suffering.

Rat primary cortical cells were isolated from the neonatal cortex from post-natal day (PND) 0–1 Wistar rat pups as described previously (Kasteel and Westerink, 2017; Gerber et al., 2021), with minor modifications. Briefly, rat pups were decapitated and cortices were rapidly dissected on ice in dissection medium (Neurobasal®-A supplemented with sucrose [25 g/L], L-glutamine [450 μM], glutamate [30 μM], penicillin/ streptomycin [1 %], and FBS [10 %], pH 7.4) during the entire isolation.

Cells were seeded (50 μL droplet containing 2×10^6 cells/mL, i.e., 1×10^5 cells/well) in dissection medium on 0.1 % PEI-coated (24 mM sodium borate/50 mM boric acid in Milli-Q adjusted to pH 8.4 diluted in borate buffer) 48-well MEA plates (Axion Biosystems Inc., Atlanta, USA). After seeding, plates were kept at 37 °C in a 5 % CO_2 atmosphere in a humidified incubator. Cells were allowed to adhere for at least 2 h, following which 450 μL dissection medium per well was added. After 1 day in culture (DIV1), 90 % of the dissection medium was replaced with glutamate medium (Neurobasal®-A supplemented with sucrose [25 g/L], L-glutamine [450 μM], glutamate [30 μM], penicillin/streptomycin [1 %], and B-27 supplement [2 %], pH 7.4). At DIV4, 90 % of the medium was replaced with FBS medium (Neurobasal®-A supplemented

with sucrose [25 g/L], L-glutamine [450 μM], penicillin/streptomycin [1 %], and FBS [10 %], pH 7.4). See Table 1 for details on the different cell populations in the model.

2.2.2. SynFire® iNs co-culture

SynFire® glutamatergic neurons, SynFire® GABAergic neurons and SynFire® astrocytes (all from NeuCyte, Sunnyvalle, CA, USA) were thawed and cultured according to the manufacturer's protocol and as described previously (Tukker et al., 2020). In short, each cell type was thawed separately in DMEM-F12 medium. The cell pellet was dissolved in complete seeding medium (containing seeding supplement) at a density of 10k cells/μL (for all cell types). Next, a mixture was made containing 140k glutamatergic neurons (52 % of total cell number), 60k GABAergic neurons (22 %) and 70k astrocytes (26 %). The mixture was plated in 50 μL droplets (270k cells in total) over the electrode field of pre-coated MEA wells and cells were left overnight to adhere. On the next day (DIV1), 250 μL RT complete short-term maintenance medium (containing short-term supplement) was added. At DIV3 and DIV5, 50 % medium changes with complete short-term maintenance medium took place. The 50 % medium changes at DIV7, 10, 13, 16, 19, 22 and 25 were performed with RT complete long-term maintenance medium (containing long-term supplement A and B). See Table 1 for details on the different cell populations in the model.

2.3. Multi-well microelectrode array (mwMEA) recordings

Multi-well microelectrode array (mwMEA) plates contain 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 that can be recorded simultaneously (Axion Biosystems Inc., Atlanta, USA). Spontaneous electrical activity was recorded as described previously (Kasteel and Westerink, 2017; Gerber et al., 2021). Briefly, spontaneous electrical activity was recorded using a Maestro MEA platform with integrated heating system and temperature controller using Axion's Integrated Studio software (AxIS 2.4.2.13). To obtain raw data files, channels were sampled simultaneously at a constant temperature of 37°C with a gain of 1200 × and a sampling frequency of 12.5 kHz/channel using a band-pass filter (200–5000 Hz).

Experiments were performed at DIV9–11 (rat cortical cultures, for each experimental condition, cultures from at least 2 different isolations/seedings were used) or at DIV28–38 (SynFire® iNs co-cultures, single seeding for each experimental condition). Exposure took place when cultures exhibited signs of mature networks, such as burst and network bursting behaviour (also see Fig. S1). All plates contained medium and solvent controls and all recordings were performed at 37°C. Plates were allowed to equilibrate for ~5 min prior to every recording. In order to investigate the acute effects of the test compounds, first a 30 min baseline recording of the spontaneous activity was performed. Next, cells were exposed (1:10 dilution) and a 30 min exposure recording was made. Each well was exposed to a single test condition to prevent potential effects of cumulative dosing, like receptor (de)sensitisation. In case of co-exposure, wells were exposed to TTX and the pharmaceutical at exactly the same time in a mixture. Mixtures were made immediately before exposure. Stock solutions of compounds dissolved in DMSO or demineralised water were pre-diluted in the appropriate culture medium just prior to the experiment (solvent concentration never exceeded 0.1 % v/v). Based on the data obtained in rat cortical cultures, four drugs were selected for the experiments in human neuronal co-cultures. Riluzole, Lidocaine, Flecainide and Carbamazepine were selected since their calculated IC_{50} values in rats were comparable to detected plasma levels of these drugs in humans (Table 2).

Table 1
Details of the cell populations within the culture models.

Culture	Astrocytes / neuron	Ratio excitatory / inhibitory neurons
Rat primary cortical culture	45 % / 55 % Gortz et al., (2004);Tukker et al., (2016)	70–80 % glutamatergic, 20–30 % GABAergic (Markram et al., 2004)
SynFire® iNs co-culture	26 % / 74 %	70 % glutamatergic, 30 % GABAergic

Source:Adapted from Tukker et al. (2020).

2.4. Data analysis and statistics

To analyse the effect of the test compound on spontaneous neuronal activity, raw data files were re-recorded to obtain.spk files for further data analysis. During the re-recording, spikes were detected using the AxIS spike detector (Adaptive threshold crossing, Ada BandFlt v2) with a variable threshold spike detector set at 7 × (rat cortical culture) or 5.5 × (SynFire® iNs co-cultures) standard deviation (SD) of the internal noise level (rms) on each electrode. Post/pre-spike duration was set to 3.6/2.4 ms. Next,.spk files were loaded in NeuralMetric Tool (version 2.2.4 Axion BioSystems) to obtain average mean spike rate (MSR; spikes/s) of all active electrodes (>0.01 spike/s) of each active well (≥ 1 active electrode).

The effects of test compounds on spontaneous activity were 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 (TR) by ($MSR_{exposure}/MSR_{baseline}$) x 100 %. TRs were normalised against appropriate solvent controls: medium (Valproic acid and Lidocaine), demineralised water (TTX) or DMSO (Chloroquine, Carbamazepine, Lamotrigine, Mexiletine, Flecainide, Fluoxetine and Riluzole) controls.

All data are presented as mean ± SEM from the number of wells (*n*) and plates (*N*) indicated. Outliers in control and effect data (± 2xSD) were removed (<5 %). Concentration response relationships were analysed using the PROAST package (version 70.3) operating in R version 3.6.0. This software allows for testing of several exponential models. Based on the AIC (Akaike information criterion; estimator for relative of information lost for any given model) the best model out of the set was selected and used for estimation of IC_{50}, IC_{20} and 'No-Observed-Effect-Concentrations' (NOEC). Benchmark response (BMR) cut-offs were based on the average variation from control wells from rat cortical and human iPSC-derived neuronal co-cultures and amount to 36 % ($n = 317$) and 39 % ($n = 34$), respectively. BMRs are show in the graphs as rounded values (35 % and 40 %, respectively). Effects smaller than the BMR are considered to be of limited toxicological relevance. Mixture experiments were statistically analysed by performing a two-way ANOVA analysis using the Tukey Multiple comparisons test in Graphpad Prism (version 9.0).

3. Results

3.1. The effects of TTX and drugs acting on TTX-sensitive NaV channels on spontaneous neuronal activity of rat cortical cultures

Tetrodotoxin (TTX) effectively inhibits spontaneous neuronal activity in rat primary cortical cultures, with NOEC, IC_{20} and IC_{50} values for inhibition of MSR of respectively 0.25, 0.83 and 2.11 nM (Fig. 1; n = 19–24; $N = 3$).

In addition to TTX, several drugs that are known to target TTX-sensitive Na_V channels were screened for their effects on neuronal activity (Fig. 2). Riluzole potently inhibits spontaneous neuronal activity, with an IC_{50} for inhibition of MSR of 1.2 μM ($n = 24$–30; $N = 4$–6). Chloroquine also inhibits spontaneous neuronal activity, although less potently with an IC_{50} for inhibition of MSR of 14.7 μM ($n = 20$–30; $N = 4$–5), whereas Fluoxetine inhibits spontaneous neuronal activity with an IC_{50} of 4.3 μM ($n = 16$–45; $N = 3$–8).

Additionally, Valproic acid acts as a weak inhibitor of spontaneous neuronal activity, with an IC_{50} for inhibition of MSR of 2450 μM

Table 2
Summary table highlighting calculated NOEC, IC_{20} and IC_{50} values for inhibition of spontaneous neuronal activity in rat primary cortical cultures, hiPSC-derived neuronal co-cultures and reported human plasma levels.

Compound	Na_V channels	NOEC (rat)	IC_{20} (rat)	IC_{50} (rat)	NOEC (human)	IC_{20} (human)	IC_{50} (human)	Reported Plasma levels (human)
TTX	TTX-sensitive	0.25 nM	0.83 nM	2.11 nM	0.7 nM	1.39 nM	2.43 nM	
Riluzole	TTX-sensitive	0.08 μM	0.4 μM	1.2 μM	0.29 μM	0.94 μM	2.38 μM	1.84 μM (Groeneveld et al., 2001)
Chloroquine	TTX-sensitive	1.7 μM	5.7 μM	14.7 μM	–	–	–	0.63 – 2.5 μM (Frisk-Holmberg et al., 1979)
Fluoxetine	TTX-sensitive	2.2 μM	3.2 μM	4.3 μM	–	–	–	97 nM (Aronoff et al., 1984)
Valproic acid	TTX-sensitive	136 μM	696 μM	2450 μM	–	–	–	300–600 μM (Henriksen and Johannessen, 1982)
Lamotrigine	TTX-sensitive	17.0 μM	25.3 μM	35.8 μM	–	–	–	3–12 μM (Ramsay et al., 1991)
Lidocaine	TTX-sensitive	1.57 μM	5.97 μM	16.7 μM	2.86 μM	11.26 μM	32.5 μM	4–10 μM (Cohen et al., 1972)
Mexiletine	TTX-resistant	3.52 μM	8.97 μM	18.5 μM	–	–	–	11 μM (Haselbarth et al., 1981)
Flecainide	TTX-resistant	0.99 μM	3.08 μM	7.43 μM	0.35 μM	1.18 μM	3.07 μM	0.7 – 4.8 μM (Tjandra-Maga et al., 1986)
Carbamazepine	TTX-resistant	5.1 μM	15.8 μM	37.8 μM	1.52 μM	6.30 μM	18.95 μM	24 μM (Grimsley et al., 1991)

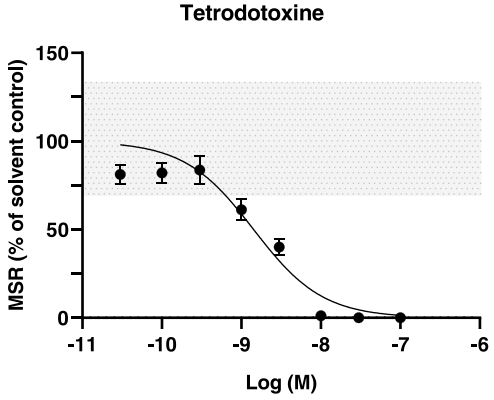

Fig. 1. Concentration-response curve of TTX on spontaneous neuronal activity (expressed as Mean Spike Rate (MSR) as percentage of solvent control) in rat primary cortical cultures. Data points represent mean ± SEM. The grey shaded area represents a benchmark response of 35 %, which is derived from the average variation in control experiments.

($n = 16$–20; $N = 3$–4). Lamotrigine inhibits spontaneous neuronal activity with an IC_{50} of 35.8 μM ($n = 19$–20; $N = 3$–4), whereas Lidocaine ($n = 18$–20; $N = 3$–4) inhibits spontaneous neuronal activity with an IC_{50} of 16.7 μM. All calculated NOEC, IC_{20} and IC_{50} values for TTX and these drugs are shown in Table 2.

3.2. Mixture experiments of TTX with drugs acting on TTX-sensitive NaV channels in rat cortical cultures

In order to determine if pharmacodynamic interactions occur between TTX and these drugs on spontaneous neuronal activity, rat cortical cultures were exposed to mixtures of each individual drug with TTX at the calculated NOEC, IC_{20} and IC_{50} (Fig. 3). Additionally, cultures were exposed to just the calculated NOEC, IC_{20} and IC_{50} of the drug or TTX. At the calculated NOEC, IC_{20} and IC_{50}, TTX inhibits spontaneous neuronal activity with 0 %, 3 % and 39 %, respectively ($n = 21$–78, $N = 10$). At the calculated NOEC, IC_{20} and IC_{50}, Riluzole alone inhibits spontaneous neuronal activity with 13 %, 14 % and 43 %, respectively ($n = 16$–23, $N = 3$–4). Chloroquine alone increases neuronal activity with 34 % (NOEC) and 42 % (IC_{20}), or inhibits with 27 % (IC_{50}) ($n = 22$, $N = 2$–3). Fluoxetine alone at the calculated NOEC, IC_{20} and IC_{50} decreases spontaneous neuronal activity with 25 %, 24 % and 44 %, respectively ($n = 16$–23, $N = 3$–4). In addition, at the calculated NOEC, IC_{20} and IC_{50}, Valproic acid alone inhibits spontaneous neuronal activity with 0 %, 11 % and 31 %, respectively ($n = 16$–22, $N = 2$–3), Lamotrigine alone with 25 %, 33 % and 18 %, respectively ($n = 13$–23, $N = 2$–4) and Lidocaine alone with 4 %, 7 % and 31 %, respectively ($n = 15$–16, $N = 2$–3).

Mixture effects of TTX with drugs acting on TTX-sensitive Na_V channels are considered to be additive, only when they significantly decrease spontaneous neuronal activity compared to both compounds individually. In combination with TTX, an additive effect for Riluzole was only seen at calculated IC_{20} levels, which significantly increases the inhibition of neuronal activity compared to TTX and Riluzole alone. Similarly, additive effects were found for the combinations of Fluoxetine and Lidocaine with TTX at calculated IC_{20} and calculated IC_{50} levels, whereas for Lamotrigine, Chloroquine and Valproic acid with TTX additive effects were observed only at calculated IC_{50} levels.

3.3. The effects of drugs targeting TTX-resistant NaV channels on spontaneous neuronal activity of rat cortical cultures

Mexiletine ($n = 19$–23; $N = 3$–4), Flecainide ($n = 19$–26; $N = 3$–4) and Carbamazepine ($n = 14$–20; $N = 3$–4) all act as inhibitors of spontaneous neuronal activity (Fig. 4), with IC_{50}'s for inhibition of MSR of 18.5 μM, 7.4 μM and 37.8 μM, respectively. Their calculated NOEC, IC_{20} and IC_{50} values are shown in Table 2.

3.4. Mixture experiments of TTX with drugs acting on TTX-resistant NaV channels in rat cortical cultures

When cells were exposed to the calculated NOEC, IC_{20} and IC_{50} for Mexiletine alone, spontaneous neuronal activity is inhibited with 26 %, 22 % and 56 %, respectively ($n = 13$–25, $N = 2$–4). Flecainide alone at the calculated NOEC, IC_{20} and IC_{50} inhibits spontaneous neuronal activity with 23 %, 10 % and 47 %, respectively ($n = 19$, $N = 3$). Finally, Carbamazepine alone at the calculated NOEC, IC_{20} and IC_{50} inhibits spontaneous neuronal activity with 32 %, 1 % and 45 %, respectively ($n = 22$, $N = 3$–4).

In combination with TTX, an additive effect was seen for all three drugs at calculated IC_{20} and IC_{50} levels, where the inhibition of spontaneous neuronal activity is significantly increased compared to the inhibition by the drugs alone (Fig. 5).

3.5. The effects of TTX and drugs acting on TTX-sensitive and TTX-resistant Na_V channels on spontaneous neuronal activity of human neuronal co-cultures

In order to determine interspecies differences, the effects of TTX and a selection of four drugs (Riluzole, Lidocaine, Flecainide and Carbamazepine) on spontaneous neuronal activity were investigated using human iPSC-derived co-cultures of neurons and astrocytes. TTX acts as a potent inhibitor of spontaneous neuronal activity in these human neuronal co-cultures (Fig. 6), with an IC_{50} for inhibition of MSR of 2.4 nM ($n = 6$–7; $N = 1$). Riluzole inhibits spontaneous neuronal activity with an IC_{50} of 2.4 μM ($n = 4$–8; $N = 1$), whereas Lidocaine inhibits spontaneous neuronal activity with an IC_{50} of 32.5 μM ($n = 8$; $N = 1$). Flecainide inhibits spontaneous neuronal activity with an IC_{50} of 3.07 μM ($n = 6$; $N = 1$), whereas Carbamazepine inhibits spontaneous

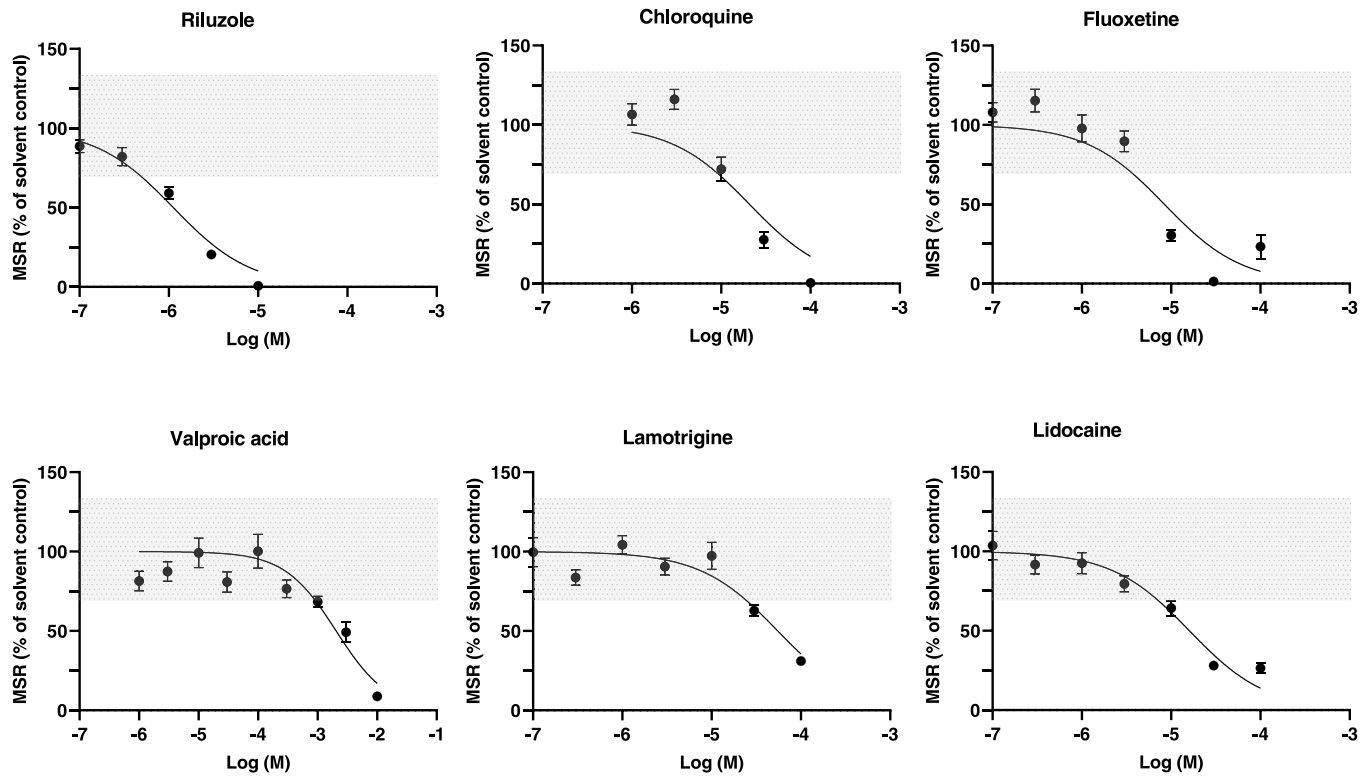

Fig. 2. Concentration-response curve of drugs acting on TTX-sensitive Na_V channels on spontaneous neuronal activity (expressed as Mean Spike Rate (MSR) as percentage of solvent control) in rat primary cortical cultures. Data points represent mean ± SEM. The grey shaded area represents a benchmark response of 35 %, which is derived from the average variation in control experiments.

neuronal activity with an IC_{50} of 18.95 μM ($n = 5$–12; $N = 1$). All calculated NOEC, IC_{20} and IC_{50} values for TTX and these drugs are shown in Table 2.

3.6. *Mixture experiments of TTX with drugs acting on TTX-sensitive and TTX-resistant Na_V channels in human neuronal co-cultures*

When cells were exposed to the calculated NOEC, IC_{20} and IC_{50} for TTX alone, spontaneous neuronal activity is inhibited with − 4%, 17 % and 39 %, respectively ($n = 6$, $N = 1$). When cells were exposed to the calculated NOEC, IC_{20} and IC_{50} for Riluzole alone, spontaneous neuronal activity is inhibited with 16 %, 22 % and 43 %, respectively ($n = 6$, $N = 1$). Mixture experiments of TTX with Riluzole at the calculated NOEC, IC_{20} and IC_{50} ($n = 6$–7, $N = 1$) only show an additive effect at the calculated IC_{20} concentrations. Lidocaine alone at the calculated NOEC, IC_{20} and IC_{50} inhibits spontaneous neuronal activity with 52 %, 10 % and 91 %, respectively ($n = 6$, $N = 1$). No additive effects are observed for the combinations of Lidocaine with TTX at the tested concentrations. Furthermore, Flecainide alone at the calculated NOEC, IC_{20} and IC_{50} inhibits neuronal activity with 27 %, 48 % and 82 %, respectively ($n = 6$, $N = 1$). No additive effects are observed for the combinations of Flecainide with TTX at the tested concentrations. Finally, Carbamazepine alone inhibits spontaneous neuronal activity with − 7 %, 35 % and 51 %, respectively ($n = 6$, $N = 1$). No additive effects are observed for the combinations of Carbamazepine with TTX at the tested concentrations (Fig. 7).

4. Discussion

The present study investigated a potential pharmacodynamic interaction between the marine neurotoxin TTX and several medicinal drugs. TTX potently inhibits spontaneous neuronal activity (IC_{50} ~2 nM) in rat primary cortical cultures and hiPSC-derived neuronal co-cultures, in line with earlier findings (Nicolas et al., 2014). We used the same procedure in the present study to determine the inhibitory potential of a selection of medicines, which are known to act on TTX-sensitive and TTX-resistant Na_V channels, on spontaneous neuronal activity. Additionally, potential additivity of the inhibitory effects of TTX by these drugs was assessed. Our data reveal a clear pharmacodynamic interaction between TTX and medicines acting on TTX-sensitive and TTX-resistant Na_V channels in rat cortical neurons.

When comparing the sensitivity of rat cortical cultures to human iPSC-derived neuronal co-cultures, it becomes evident that TTX is equipotent in both cell types with IC_{50} values of 2.1 and 2.4 nM for rat cortical cultures and human iPSC-derived neuronal co-cultures, respectively. These results confirm good reproducibility of the current *in vitro* approach as well as limited interspecies differences for TTX-induced inhibition of Na_V channels. These limited interspecies differences have previously also been observed in *in vivo* animal studies with various mammalian species on the lethality of TTX (Kao, 1966), which is likely explained by the high conservation of the Na_V channels between mammalian species (Goldin et al., 2000; Lopreato et al., 2001). Although the current *in vitro* approach cannot fully replace *in vivo* studies due to the lack of pharmacokinetic processes, the limited interspecies differences between the lethality and inhibitory potency of TTX highlight the potential of this approach for human risk assessment of TTX.

With respect to the risk assessment of TTX, there is a general lack of data regarding exposure levels of TTX in human cases of intoxications. This hampers risk assessment on TTX using human data and it therefore seems essential to use animal data. Over the years, an increasing amount of information has been gathered regarding the toxicological mechanisms and effects of TTX in different animal species. We previously used the available animal data to estimate an acute reference dose (1.33 μg/kg) for TTX (Kasteel and Westerink, 2017). When setting the acute reference dose, three uncertainty factors were applied, including one accounting for human risk groups that could be more sensitive to

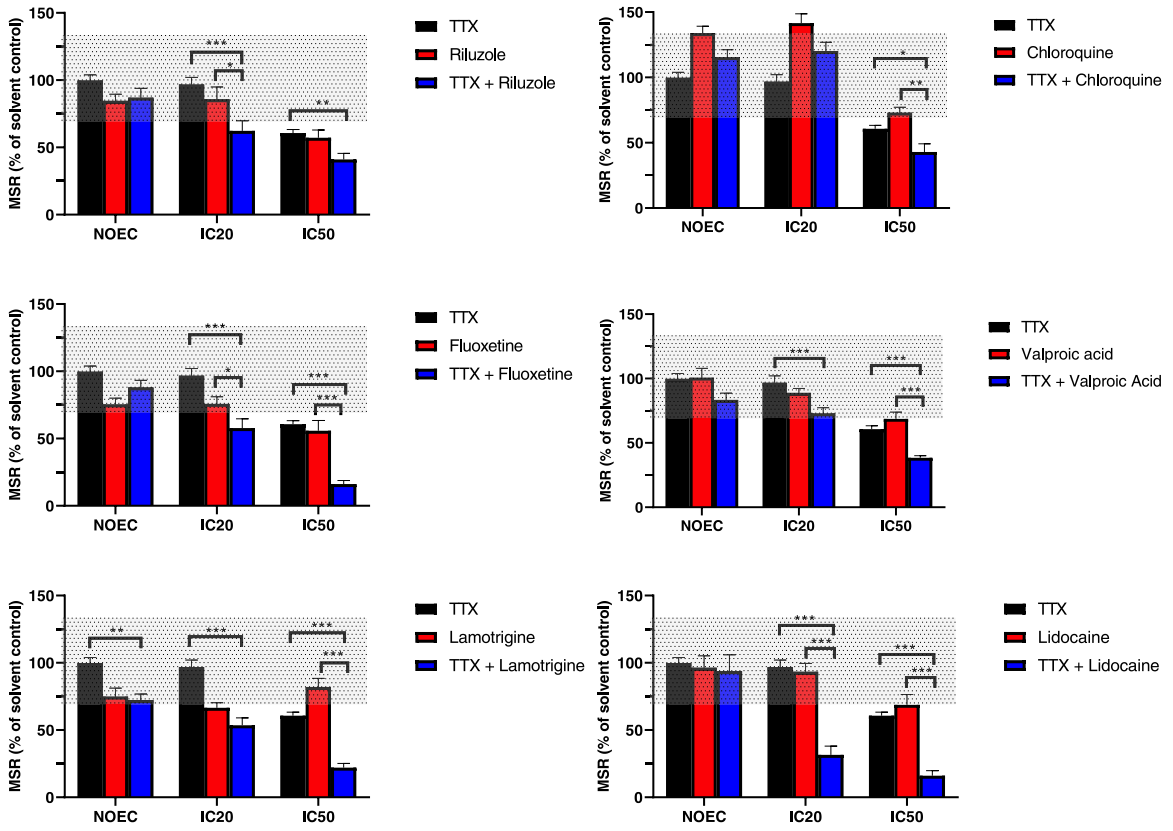

Fig. 3. Bar graphs depicting the effect on spontaneous neuronal activity in rat primary cortical cultures exposed to calculated NOEC, IC_{20} and IC_{50} levels of TTX (black), drugs acting on TTX-sensitive Na_V channels (red) and the binary combination of TTX with drug (blue). See Supplementary Table S1 for exact values. Bars depict Mean Spike Rate (as percentage of solvent control) and represent mean ± SEM (n = 15–36; N = 2–5). * , $p < 0.05$; * *, $p < 0.005$; * ** , $p < 0.001$. The grey shaded area represents a benchmark response of 35 %, which is derived from the average variation in control experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

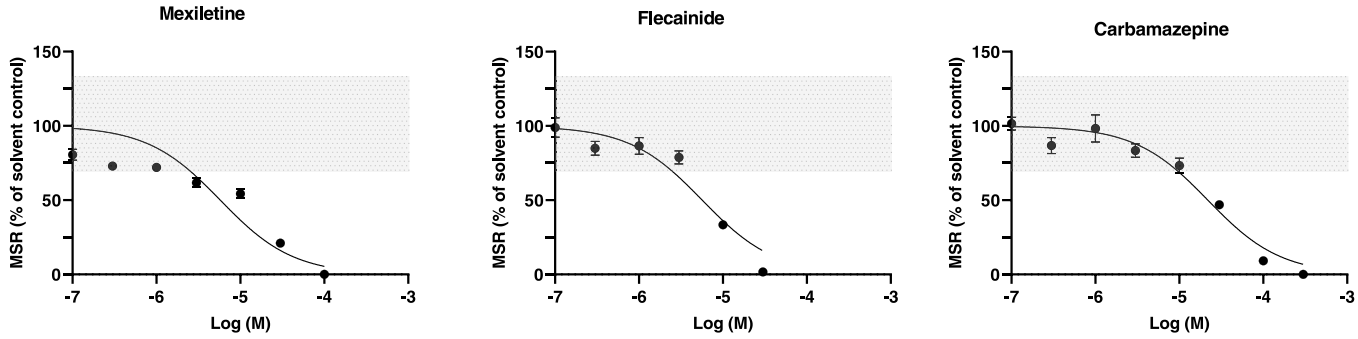

Fig. 4. Concentration-response curve of drugs acting on TTX-resistant Na_V channels on spontaneous neuronal activity (expressed as Mean Spike Rate (MSR) as percentage of solvent control) in rat primary cortical cultures. Data points represent mean ± SEM. The grey shaded area represents a benchmark response of 35 %, which is derived from the average variation in control experiments.

TTX-induced toxicity. More susceptible risk groups generally comprise of individuals with altered toxicokinetics (e.g., reduced kidney function) and individuals that are more prone to the toxicodynamic effects of the particular toxicant. The latter may be caused by the concomitant exposure to compounds, such as pharmaceuticals, with a similar toxicodynamic profile, possibly leading to a pharmacodynamic interaction. As such, the use of medicines acting on Na_V channels could potentially increase the sensitivity to the toxic effects of TTX.

Various medicines are known to target Na_V channels. The present study has selected a range of medicines that target TTX-sensitive and TTX-resistant Na_V channels and assessed their potency for inhibiting spontaneous neuronal activity (Table 2). All drugs concentration-dependently decrease the activity of rat primary cortical neurons, but with widely varying inhibiting potencies (NOEC, IC_{20}, IC_{50}). This is in line with the pharmacodynamic profile of these drugs (Doble, 1996; Thanacoody, 2016; Poulin et al., 2014; Farber et al., 2002; Muroi and Chanda, 2009; Chinushi et al., 2003; Liu et al., 2003; Lipkind and Fozzard, 2010). For Riluzole, Mexiletine, Flecainide and Carbamazepine, the calculated IC_{50} values are within the range of human plasma concentrations. This means that inhibition of neuronal activity is expected to also occur clinically in patients using these medicines. For some of these drugs, the potential to inhibit neuronal activity relates well to their clinical use and their desired pharmacological effects. Riluzole is a neuroprotective agent and a known sodium channel blocker used in the

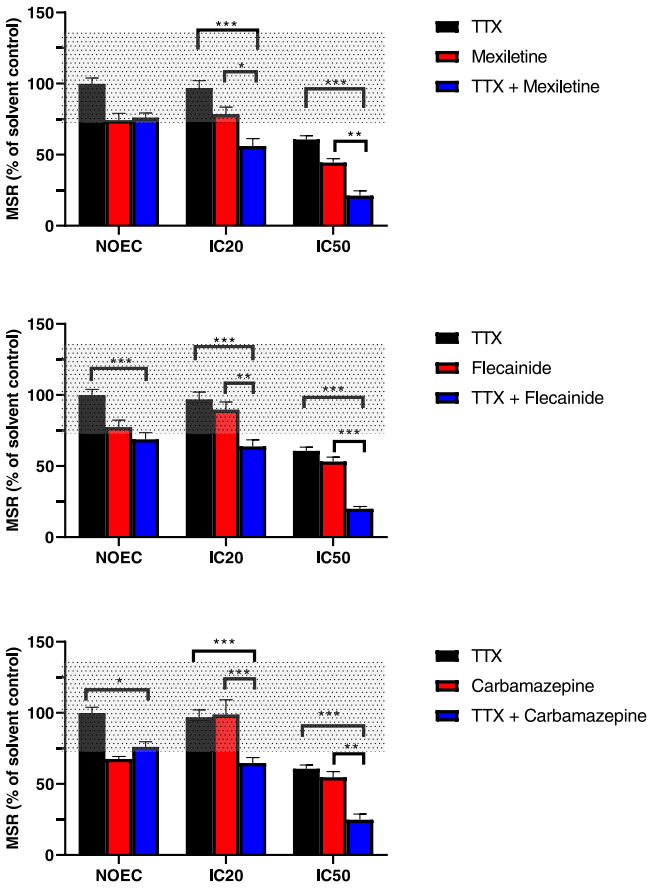

Fig. 5. Bar graphs depicting the effect on spontaneous neuronal activity in rat primary cortical cultures exposed to calculated NOEC, IC_{20} and IC_{50} levels of TTX (black), drugs acting on TTX-resistant Na_V channels (red) and the binary combination of TTX with drug (blue). See Supplementary Table S1 for exact values. Bars depict Mean Spike Rate (as percentage of solvent control) and represent mean ± SEM (n = 13–24; N = 2–3). * , $p < 0.05$; * *, $p < 0.005$; * ** , $p < 0.001$. The grey shaded area represents a benchmark response of 35 %, which is derived from the average variation in control experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treatment of amyotrophic lateral sclerosis (ALS). In addition, Carbamazepine is a well-known voltage-gated sodium channel blocker used to treat epilepsy. However, for the other medicines, inhibition of neuronal activity at clinically relevant concentrations may contribute to an increased risk for neurotoxicological effects. Mexelitine and flecainide are antiarrhythmic agents that inhibit inward sodium currents by blocking sodium channels ($Na_V1.5$) that are needed for the initiation and conduction of action potentials. For Chloroquine, Valproic acid, Lamotrigine and Lidocaine, the calculated IC_{50} values are within one order of magnitude of plasma concentrations. Only for Fluoxetine, the calculated NOEC and IC_{50} values appears of limited relevance for human toxicology considering the much lower plasma concentrations.

The medicines with calculated IC_{50} values within the range of reported human plasma concentrations (Riluzole, Mexiletine, Flecainide and Carbamazepine) were additionally assessed for their ability to inhibit spontaneous neuronal activity in human iPSC-derived neuronal co-cultures. In general, these medicines were roughly equipotent in both rat and human neuronal cultures, which is represented by the limited difference (~2x) in calculated NOEC, IC_{20} and IC_{50} values. Notably, the medicines that act on TTX-sensitive sodium channels (Riluzole, Lidocaine) seem more potent in rat primary cortical cultures, whereas those that act on TTX-resistant sodium channels (Flecainide/Carbamazepine) seem more potent in human iPSC-derived neuronal co-cultures. While experimental evidence is currently lacking, it is tempting to speculate that this could be explained by differences in the ratio of expression of TTX-sensitive and TTX-resistant sodium channels between these model systems.

Given the steepness of the concentration-effect curve of TTX for inhibition of neuronal activity, already a modest augmentation of TTX-induced inhibition at calculated IC_{20} or IC_{50} levels by medicines upon concomitant exposure could potentially result in a full inhibition of action potential generation and propagation. This would severely increase an individual's sensitivity to the toxicity of TTX when using one of these medicines.

The present study therefore exposed rat and human neuronal co-cultures to mixtures of TTX and the selected medicines (at calculated NOEC, IC_{20} and IC_{50} levels) and investigated a potential pharmacodynamic interaction between them. As stated before, the mixture effects are only considered to be additive, when they significantly decrease spontaneous neuronal activity compared to both compounds individually.

For data derived from rat primary cortical cultures, we generally did not observe additivity at calculated NOEC levels, whereas binary mixtures at calculated IC_{20} and IC_{50} values mostly show additivity. All medicines acting on TTX-sensitive Na_V channels significantly increased the inhibitory effect of TTX on spontaneous neuronal activity, either at IC_{20}, IC_{50} or at both levels. Additionally, medicines targeting TTX-resistant Na_V channels all significantly increased the inhibitory effect of TTX at both the calculated IC_{20} and IC_{50} levels. These data highlight that the use of pharmaceuticals could increase human sensitivity to the toxicological effects of TTX. This raises the importance of identifying and including vulnerable risk groups in the risk assessment of TTX for instance by applying additional safety factors.

The data set obtained with rat primary cortical cultures is very solid with data generally derived from 3 independent experiments with ~20 wells per condition. Even with the high number of replicates in the rat data set, the variation in these data is occasionally relatively high (up to 25 %). This degree of variation in the MEA experiments also explains why the calculated effect concentrations (NOEC, IC_{20}, IC_{50}) not fully match with the measured degree of inhibition at these concentrations, which can hamper interpretation. Such high variation is standard to MEA experiments and likely, at least partly, due to the self-organising properties of the neuronal networks as well as the high degree of integration of the read out (neuronal activity).

In contrast to the data derived from rat cultures, the data from hiPSC-derived neuronal co-cultures is generally derived from a single plating, with ~6 replicates per condition, as a result of the high costs of human iPSC-derived cells. While earlier power analysis indicated that $n > 4$ provides adequate reliability for prioritisation purposes if the effect size is considerable (Cotterill et al., 2016), the relatively low number of replicates for the human cultures occasionally yields a high degree of variation (sometimes up to 50 %). Although some additional power is provided by the number of different concentrations in case of curve fitting, some caution should therefore be taken when interpreting the data obtained from hiPSC-derived co-cultures.

Using human neuronal co-cultures only one case of additivity was found, namely for the binary mixture of Riluzole with TTX at the IC_{20} level. This could be explained by physiological (e.g., different expression ratio of TTX-sensitive and TTX-resistant channels) and methodological (i.e. small sample size in combination with large variation in the data) factors. Without further expanding the current data set from hiPSC-derived neuronal co-cultures, it is unfortunately not possible to draw any solid conclusions about the combinatory effects of the medicines and TTX on these cells.

In conclusion, this study shows that the selected medicines acting on TTX-sensitive and TTX-resistant Na_V channels all inhibit spontaneous neuronal activity in rat and human neurons. Moreover, all of these medicines significantly increase the inhibitory effect of TTX on spontaneous neuronal activity of rat primary cortical cells *in vitro*. Our data

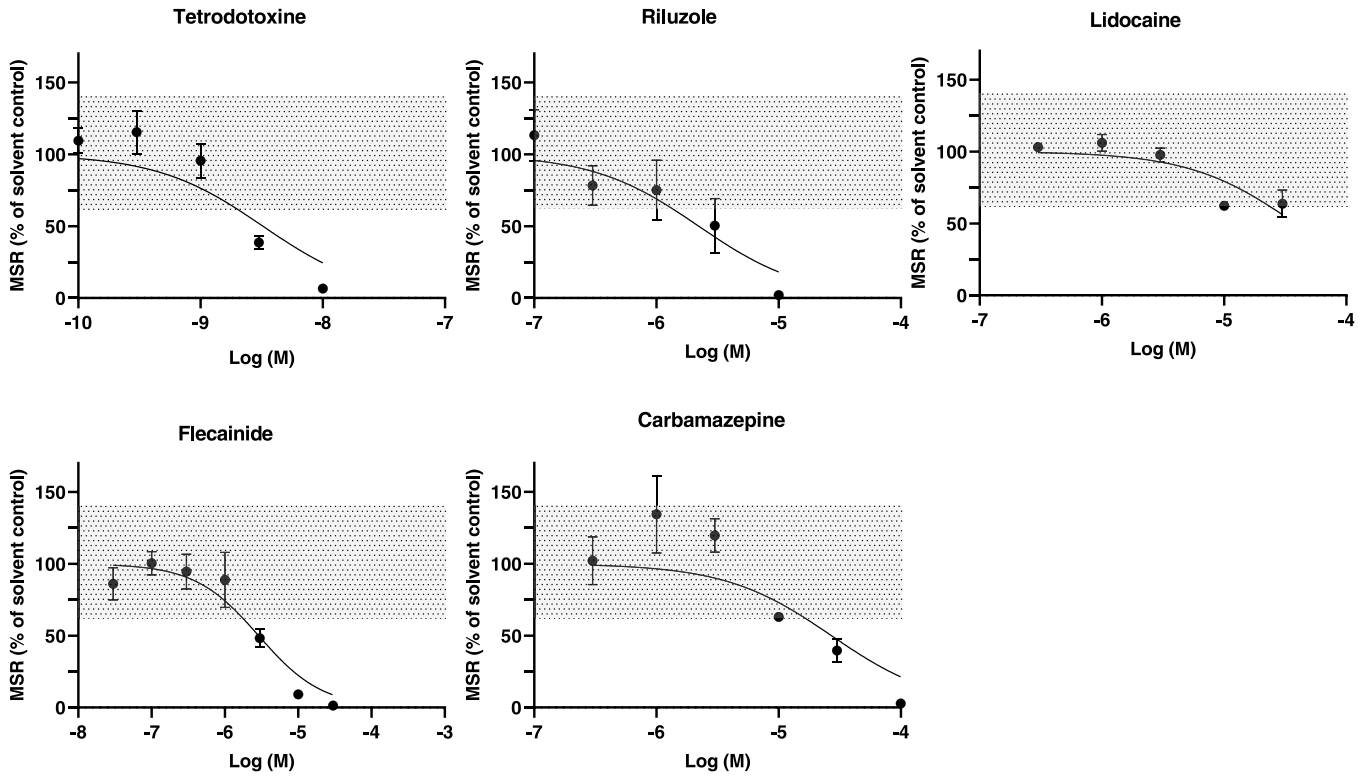

Fig. 6. Concentration-response curve of TTX and drugs acting on either TTX-sensitive or TTX-resistant Na_V channels on spontaneous neuronal activity (expressed as Mean Spike Rate (MSR) as percentage of solvent control) in human iPSC-derived neuronal co-cultures. Data points represent mean ± SEM. The grey shaded area represents a benchmark response of 40 %, which is derived from the average variation in control experiments.

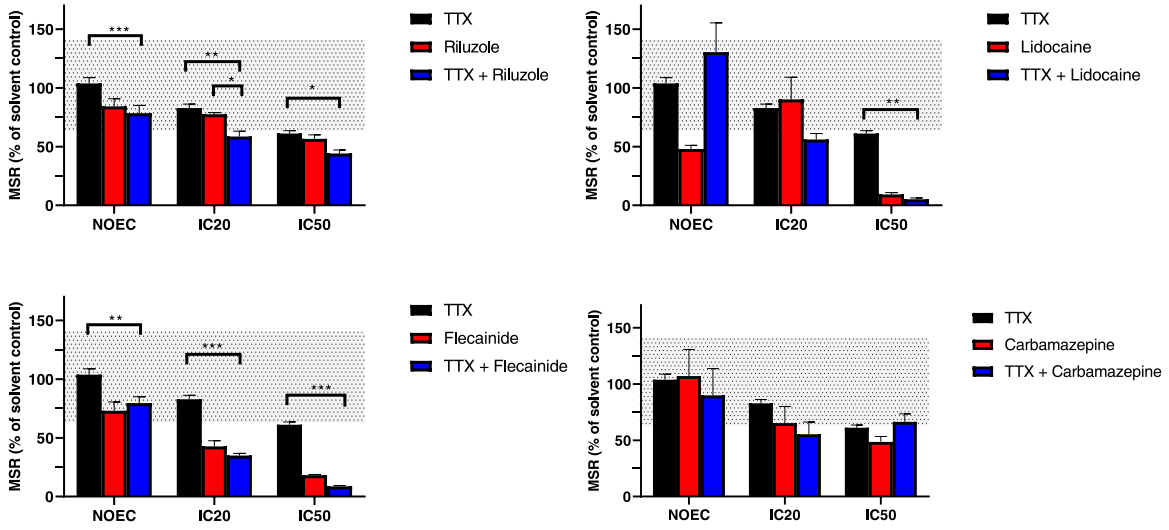

Fig. 7. Bar graphs depicting the effect on spontaneous neuronal activity in human iPSC-derived neuronal co-cultures exposed to calculated NOEC, IC_{20} and IC_{50} levels of TTX (black), drugs acting on TTX-sensitive (red, top) and TTX-resistant (red, bottom) Na_V channels and the binary combination of TTX with drug (blue). See Supplementary Table S1 for exact values. Bars depict Mean Spike Rate (as percentage of solvent control) and represent mean ± SEM (n = 6–7; N = 1). * , $p < 0.05$; * *, $p < 0.005$; * ** , $p < 0.001$. The grey shaded area represents a benchmark response of 40 %, which is derived from the average variation in control experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

thus raise the importance of identifying and including vulnerable risk groups in the risk assessment of TTX, which needs to be confirmed in additional experiments on human iPSC-derived co-cultures.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Remco Westerink reports financial support was provided by NVWA.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxlet.2022.11.005.

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