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Chronic 14-day exposure to insecticides or methylmercury modulates neuronal activity in primary rat cortical cultures

Milou M.L. Dingemans, Marijke G. Schütte, Daphne M.M. Wiersma, Aart de Groot, Regina G.D.M. van Kleef, Fiona M.J. Wijnolts, Remco H.S. Westerink*

Neurotoxicology Research Group, Toxicology Division, Institute for Risk Assessment Sciences (IRAS), Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.177, NL-3508 TD Utrecht, The Netherlands

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ABSTRACT

There is an increasing demand for *in vitro* test systems to detect neurotoxicity for use in chemical risk assessment. In this study, we evaluated the applicability of rat primary cortical cultures grown on multi-well micro-electrode arrays (mwMEAs) to detect effects of chronic 14-day exposure to structurally different insecticides or methylmercury on neuronal activity (mean spike rate; MSR).

Effects of chronic exposure to α-cypermethrin, endosulfan, carbaryl, chlorpyrifos(-oxon), methylmercury or solvent control [14 days exposure, initiated after baseline recording at day *in vitro* (DIV)7] were studied in five successive recordings between DIV10 and DIV21. The results were compared to effects of acute exposure to these same compounds (activity recorded immediately after the start of exposure after baseline recording at DIV10-11).

Chronic 14-day exposure to methylmercury, chlorpyrifos and α-cypermethrin inhibited MSR, all with a lowest-observed effect concentration (LOEC) of 0.1 μM, while exposure to endosulfan increased MSR [LOEC: 1 μM]. No significant effects were observed for chlorpyrifos-oxon and carbaryl. Similar to the observations in the chronic 14-day exposure studies, MSR was inhibited by acute 30-min exposure to methylmercury, chlorpyrifos, and α-cypermethrin [LOECs: 1 μM, 10 μM, and 1 μM, respectively], whereas endosulfan increased MSR [LOEC: 0.3 μM]. While not observed in the chronic 14-day exposure study, acute exposure to chlorpyrifos-oxon and carbaryl resulted in inhibition of MSR [LOECs: 10 μM, and 100 μM, respectively]. Effects on median interspike intervals (mISI; a measure for neuronal firing pattern) were not detected following chronic 14-day or acute 30-min exposure, except for increased mISI at acute chlorpyrifos and α-cypermethrin exposures at concentrations that also inhibited MSR.

These data indicate that the effects of chronic 14-day exposures to methylmercury and insecticides at low concentrations on spontaneous neuronal activity *in vitro* can be predicted in rapid acute screening studies using mwMEAs.

1. Introduction

The main function of neurons is to collect, combine and transmit information using chemical messengers (neurotransmitters) and electrical signals (action potentials). Cellular neurotoxic mechanisms described in scientific literature comprise chemical-induced effects on many processes and molecular components underlying neuronal function (neurophysiology). These include effects on cellular homeostasis and signaling pathways, neurotransmitter release and ion channel/neurotransmitter receptor function (de Groot et al., 2013). Effects of chemicals resulting from changes in neurophysiology are often investigated in the intact

Abbreviations: ANOVA, analysis of variance; CAR, carbaryl; CP, chlorpyrifos; CPO, chlorpyrifos-oxon; CYP, α-cypermethrin; DIV, days *in vitro*; DMSO, dimethyl sulfoxide; ES, endosulfan; FBS, fetal bovine serum; ITS, integrated testing strategies; LOEC, lowest observed effect concentration; MEA, micro-electrode array; mISI, median interspike interval; MSR, mean spike rate; mwMEA, multi-well micro-electrode array; n, number of wells; NOEC, no observed effect concentration; PLL, poly-L-lysine; TR, treatment ratio (value of parameter during exposure divided by the value of parameter during baseline recording).

* Corresponding author.
E-mail addresses: m.dingemans@uu.nl (M.M.L. Dingemans), m.g.schutte@uu.nl (M.G. Schütte), d.m.m.wiersma@uu.nl (D.M.M. Wiersma), a.degroot@uu.nl (A. de Groot), g.vankleef@uu.nl (R.G.D.M. van Kleef), f.m.j.wijnolts@uu.nl (F.M.J. Wijnolts), r.westerink@uu.nl (R.H.S. Westerink).

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animal, by studying effects on neurobehavior and/or cognition (Moser, 2011). In view of ethical considerations and to expand testing capacity, rapid *in vitro* test systems are urgently needed to (partly) replace these animal tests, e.g. to prioritize for testing using the most appropriate (cognitive) tests (Vorhees and Makris, 2015). Such *in vitro* data is particularly useful in the context of integrated testing strategies (ITS) for human risk assessment in which different pieces of (toxicological) information related to effects on human health resulting from exposure to chemicals are combined (Rovida et al., 2015; Schultz et al., 2015). Using different and complementary experimental assays to obtain reproducible information (Miller, 2014), the uncertainty of the conclusions drawn will decrease, especially with regard to complex endpoints such as neurotoxicity.

Studies investigating chemical-induced effects on specific aspects of neurophysiology, e.g. using classical electrophysiology or cellular imaging techniques, usually have a low throughput and/or require training and expertise. These limitations are largely circumvented by the use of multi-well micro-electrode array (mwMEA) systems (Johnstone et al., 2010). MEAs consist of a cell culture surface with an integrated array of microelectrodes that can be used to study *in vitro* neuronal activity by measuring local field potentials (spikes) from the cells cultured on the electrode array (Johnstone et al., 2010; Spira and Hai, 2013). This approach allows the efficient collection of large amounts of electrophysiology data transferring the experimental challenges from the practical experiments to data analysis and storage. Moreover, sterile conditions can be maintained allowing repeated measurements of activity and the investigation of long-term or repeated exposures.

In the present study, we investigated the effects of chronic 14-day exposure to structurally different insecticides or the developmental neurotoxicant methylmercury on spontaneous electrical activity of primary cortical cultures. Primary cortical cultures of rat neonates were chosen as the appropriate model for this study as these have been demonstrated to reproducibly form neuronal networks and develop electrical activity *in vitro* (Wagenaar et al., 2006; Weir et al., 2015). Moreover, this heterogeneous primary *in vitro* model consists of multiple neural cell types (different subtypes of neurons and glia) and thus contains a multitude of molecular targets that may be affected by chemical exposure (Hondebrink et al., 2016; Tukker et al., 2016; Wallace et al., 2015; Weir et al., 2015). Prolonged exposure may not only acutely affect neurophysiological processes, but also result in adaptive changes, in particular in the expression of ion channels, neurotransmitter receptors and related genes. In functional neuronal networks, such acute and adaptive chemical-induced effects on neuronal ion channels and receptors will be integrated and reflected in effects on the spontaneous electrical activity.

From earlier studies it is known that insecticides and methylmercury affect many different molecular targets within the nervous system (Farina et al., 2011; Flaskos, 2012; Marrs and Maynard, 2013; Mrema et al., 2013; Soderlund, 2012). In particular methylmercury and chlorpyrifos have been extensively studied with regard to their mechanisms of action. Nevertheless, in recent years new molecular targets for insecticides have been discovered, such as voltage-gated calcium channels (Meijer et al., 2014a,b, 2015).

Despite the urgent need for *in vitro* models to study neurotoxicity and effects on neurodevelopmental processes, there is currently only one other MEA study describing a comparison between effects induced by chronic ≥28-day and acute exposure, revealing a reduced lowest observed effect concentration (LOEC) of the marine toxin domoic acid for effects on spike rate in primary cortical cultures on MEAs upon long-term exposure compared to acute exposure (Hogberg et al., 2011). To investigate whether such a decrease of LOEC, i.e. increased sensitivity of the model, with increased exposure duration also occurs for other neurotoxicants, we have compared the effects of chronic 14-day exposure to structurally different insecticides and methylmercury on electrical activity [mean spike rate (MSR) and median interspike intervals (mISI)] in primary cortical cultures with effects in rapid screening studies (acute exposure).

2. Materials and methods

2.1. Chemicals

Insecticides endosulfan (α:β 2:1; 99.9%), α-cypermethrin (99.7%), carbaryl (99.5%) chlorpyrifos (99.9%), and methylmercury(II) chloride were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Chlorpyrifos-oxon (93.5%) was obtained from AccuStandard (New Haven, USA). Stock solutions (dilution series from 100 mM) were prepared in dimethyl sulfoxide (DMSO). See Supplementary data for CAS numbers (Table S1).

2.2. Cell culture

Rat cortical cells were isolated from neonatal [postnatal day 0–1] Wistar rats. Wistar rats were obtained from Harlan Laboratories B.V. (Horst, The Netherlands). Animals were treated humanely and with regard for alleviation of suffering. All experimental procedures were performed according to Dutch law and approved by the Ethical Committee for Animal Experimentation of Utrecht University.

Rat cortical cell were cultured in 48-well MEA plates (Axion Biosystems inc., Atlanta, US) following the protocol of McConnell et al. (2012) with minor modifications (described previously in de Groot et al., 2016; Nicolas et al., 2014). Briefly, cortices were collected by dissection on ice, dissociated to a single-cell suspension by mincing with scissors, trituration and filtering through a 100 μm mesh (EASYstrainer, Greiner). Cell suspension (50 μl/well containing $2*10^6$ cells/ml) was placed on the electrode fields in poly-L-lysine (PLL)-coated MEAs and cells were left to adhere for 2 h before adding Neurobasal-A medium (GIBCO) containing sucrose (25 g/l; Sigma), glutamine (450 μM, GIBCO), glutamate (30 μM, Sigma), penicillin/streptomycin (1%; Sigma) and fetal bovine serum (FBS; 10%; GIBCO), pH 7.4. Glial overgrowth is prevented by supplementing the cultures with glutamate at DIV1-4. The day following the cell plating (day *in vitro*; DIV 1) 90% of the medium was replaced with Neurobasal-A medium in which FBS was replaced with B-27 supplement (2%; GIBCO). At DIV4, 90% of the medium was replaced with glutamate-free Neurobasal-A medium with FBS (10%) and the cells were maintained in this medium, of which 90% was replaced weekly during the remaining culture period (up to DIV21). For parallel cell viability assays (see paragraph 2.5 on cell viability assays), cortical cultures were grown in 96-wells plates ($3*10^4$ cells in 100 μl/well).

2.3. MEA recordings

Effects of acute 30-min and chronic 14-day exposure to insecticides and methylmercury on electrical activity were investigated in primary neonatal rat cortical cultures grown on PLL-coated mwMEA plates (Axion Biosystems Inc.). Each well of the MEA plate contains 16 nanotextured gold microelectrodes (~40–50 μm diameter; 350 μm center-to-center spacing) with 4 integrated ground electrodes, yielding a total of 768 channels per plate that can be recorded simultaneously. Spontaneous electrical activity is recorded in the Maestro MEA platform using Axion's Integrated Studio software as described previously (de Groot et al., 2014, 2016; Hondebrink et al., 2016; Nicolas et al., 2014).

2.4. Experimental conditions

For each experimental condition, primary cortical cultures from at least three different isolations were used. Every well received only one exposure (i.e. one compound at one concentration). Medium and solvent controls were included in all plates. Before each recording, MEA plates were allowed to equilibrate in the Maestro for ~5 min. Cells were chronically exposed (14-days) starting after the baseline recording at DIV7 and 30-min recordings of spontaneous activity were made at DIV10, DIV14, DIV17 and DIV21. Medium (containing chemicals or solvent DMSO) was replaced after recordings at DIV7, DIV10/11 and DIV17/18. To study acute effects, the cells were exposed immediately after the baseline recording and activity is recorded for another 30 min. Stock solutions of methylmercury, chlorpyrifos, chlorpyrifos-oxon, carbaryl, endosulfan and α-cypermethrin were prediluted in cell culture medium just prior to the experiments (solvent DMSO never exceeded 0.1% v/v).

2.5. Cell viability assays

Effects of the chemicals on cell viability were investigated in parallel cultures (24 h exposure) to estimate exposure ranges for subsequent MEA experiments as well as in the MEA plates (after the last recording) to test for potential confounding of effects on electrical activity by cytotoxicity. Effects of the tested chemicals on cell viability were investigated using the Alamar Blue and Neutral Red assays (Magnani and Bettini, 2000; Repetto et al., 2008). For the parallel cell viability experiments, cells were cultured and exposed for 24 h in normal cell culture plates (Greiner Bio-one, Solingen, Germany). Wells in which the cells were lysed using brisk pipetting with demi water were used for background values. Briefly, following exposure to the chemicals of interest, cells were incubated for approximately 30 min in 11 μM AB (Invitrogen, Breda, The Netherlands) solution in phenol-red free and serum-free medium (at 37 °C, 5% CO_2; protected from light). Conversion of AB was measured spectrophotometrically at 540/590 nm (excitation/emission) using a Tecan Infinite M1000 platereader equipped with a 10W Xenon flash light source (Tecan Group Ltd; Männedorf, Switzerland). After removal of the AB solution, cells were incubated for approximately 1 h with 100 μl NR solution (Invitrogen, Breda, The Netherlands; 12 μM in phenol-red free Neural Basal A medium). Following incubation, the NR solution was carefully removed, and cells were lysed in 100 μl extraction solution (1% glacial acetic acid, 50% ethanol, and 49% H_2O). After 30 min extraction, NR was measured spectrophotometrically at 530 and 645 nm (excitation/emission) also using a Tecan Infinite M1000 platereader. Data was processed using iControl software (version 7.1). Effects of the tested chemicals on cell viability of cortical cultures used for MEA recordings were investigated using the Alamar Blue assay only. Cells were incubated in 125 μl AB medium (12,5 μM) for 30 min. This medium was transferred to a normal 96-wells plate and AB was measured as described above.

2.6. Data analysis and statistics

Spike data were extracted from the recordings of spontaneous activity (.raw files). Spikes were defined by >7 times standard deviation of the internal noise level (rms) on each electrode. Spike frequencies, and spike timestamps from the resulting files containing spike information (.spk files) were further analyzed using NeuroExplorer® [Nex Technologies, Madison (AL), US]. Custom-made excel macros were used for further data analysis.

In the chronic 14-day exposure experiments, electrodes were included with minimum activity of 0.01 spikes/s at DIV7 to calculate treatment ratios (TRs) and well averages. TRs were calculated by normalizing the activity recordings at DIV10, DIV14, DIV17 and DIV21 to the activity at DIV7 (baseline activity recorded before the onset of exposure at DIV7). Wells were selected for data analysis if average activity within the well at DIV7 ranged between 0.05-1 spikes/s. For the analysis of acute effects only electrodes recording stable activity were included [stability is identified based on similarity in spike frequencies in six 5-min intervals of the baseline recording (with minimum baseline activity of 0.1 spikes/s)]. Baseline frequency recorded by the selected electrodes was paired with the activity recorded by the same electrodes after the onset of exposure and TRs were calculated. As transient effects were not observed, the first 10-min of the recordings were excluded from data analysis to avoid influence of potential exposure artefacts. TRs were averaged per well, normalized to the DMSO solvent control, and log-transformed for statistical analysis.

The same wells and electrodes used to determine effects of chronic (14-day) or acute (30-min) effects on spike rate were also used to calculate effects on the number of active electrodes as well as effects on the distribution of interspike intervals (ISI) derived from spike timestamps. Active electrodes were defined as electrodes on which >0.1 spikes/s were recorded within a well. In the chronic 14-day exposure setting, the number of active electrodes and number of electrodes included for the MSR data analysis (based on the inclusion criteria described above) were used to calculate percentages of active electrodes per well per time point. In the acute exposure setting, the number of active electrodes during exposure and baseline (based on the inclusion criteria described above) were used to calculate a percentage of active electrodes per well. Possible effects of chemicals on temporal patterns of electrical activity were investigated as shifts in the distribution of interspike intervals. Effects on geometric mean (per well) of mISI values (per electrode) were investigated for all exposure conditions up to DIV14 (7-days exposure) and below the LOEC (defined as the lowest concentration at which a statistically significant effects was observed) for acute effects on MSR.

Unless otherwise noted, data are presented here as average ± SEM. The exact number of wells (n) are indicated in the results and figure legends. Outlier wells in control and effect spike rate data (defined as not within average ± 2 × SD) were removed. Similarly, outliers in cell viability data and mISI were removed. Logistic concentration-response curves were fitted with a Hill slope (4 parameters) using GraphPad Prism software (v6, GraphPad Software, La Jolla CA, USA). Where applicable, one-way ANOVAs and non-parametric Friedman tests were performed in combination with *post hoc* Bonferonni tests and Bonferonni-corrected Wilcoxon signed rank tests, respectively, using SPSS 22 (SPSS, Chicago, Illinois).

3. Results

3.1. Development of spontaneous electrical activity in cortical cultures

Spontaneous electrical activity in primary rat cortical cultures develops within the first week of *in vitro* culture, reaches its maximum value around DIV10 after which the spontaneous activity gradually subsides (Figs. 1 and S1). Solvent control DMSO (0.1% v/v) does not affect the activity development in the cortical cultures. It can also be observed that the application of the additional criteria for DIV7 activity, which excludes wells with very low activity as well as wells with overly high activity at DIV7 before the start of exposure, does not affect the representation of activity development using the average MSR.

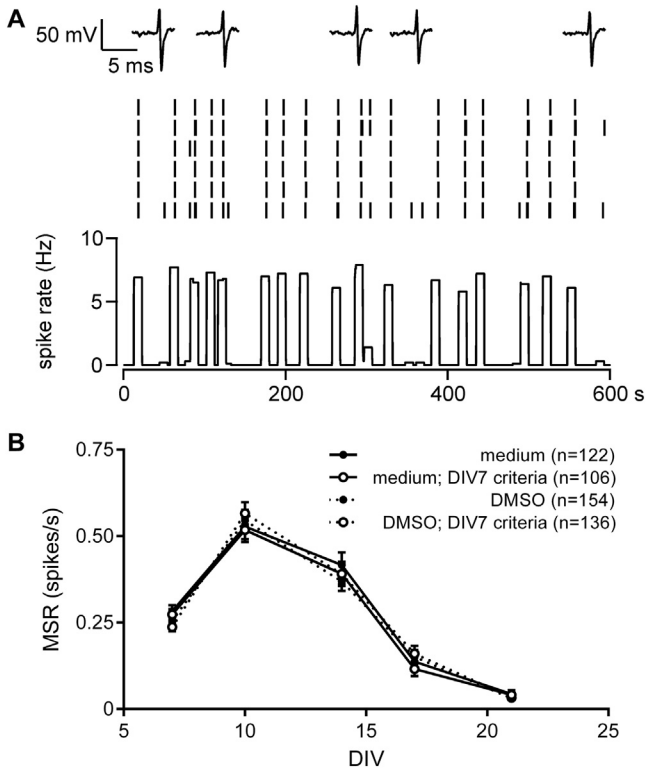

Fig. 1. A. Representative example of electrical activity recorded in a primary rat cortical culture (DIV7) on a MEA. Spike activity identified as field potential traces (top) is summarized in spike raster plots (middle) in which each mark represents one or more spikes. Each row depicts 10-min of data of individual electrodes in the well. Well activity is visualized using a frequency plot (bottom; with 0.1 s moving average in 10 s windows). B. Development of spontaneous electrical activity in primary rat cortical cultures *in vitro* (see also Fig. S1). Comparable development is observed in control cultures exposed to medium and solvent DMSO (0.1% v/v). Activity is investigated in >100 wells per condition derived from 12 different isolations (both medium and DMSO exposed wells derived from each isolation) and expressed as average ± SEM. No difference is observed if wells with divergent activity at DIV7 (MSR: <0.05 spikes/s or >1 spikes/s) are excluded from the data.

3.2. Effects of exposure to methylmercury or insecticides on spontaneous activity in primary rat cortical cultures

Baseline spontaneous electrical activity of unexposed primary cortical cultures was recorded at DIV7 after which the cultures were chronically exposed for 14-days to methylmercury or insecticides (Fig. 2 and Table 1). Methylmercury concentration-dependently inhibited the development of spontaneous electrical activity (Friedman: $P < 0.0001$) with a lowest-observed effect concentration of 0.1 μM [Wilcoxon: 0.1 μM methylmercury ($P < 0.001$) and 1 μM methylmercury ($P < 0.0001$) differ significantly from DMSO]. Inhibition of the development of spontaneous electrical activity was also observed when cultures were exposed to chlorpyrifos [Friedman: $P = 0.009$; Wilcoxon: 0.1 μM chlorpyrifos ($P = 0.005$); 1 μM chlorpyrifos ($P < 0.0001$) and 10 μM chlorpyrifos ($P = 0.004$)] or α-cypermethrin [Friedman: $P < 0.0001$; Wilcoxon: 0.1 μM α-cypermethrin ($P < 0.0001$) and 1 μM α-cypermethrin ($P < 0.0001$)]. Exposure to endosulfan also altered the development of spontaneous electrical activity (Friedman: $P < 0.0001$) with a higher activity compared to the DMSO control at the highest exposure concentration of 1 μM (Wilcoxon: $P < 0.0001$). Exposure to chlorpyrifos-oxon or carbaryl up to 10 μM did not alter the development of spontaneous electrical activity (Friedman: $P = 0{,}024$ and $P = 0.006$, respectively, but LOECs were not identified in *post hoc* analyses). No effects of chronic 7-day exposure were observed on mISI (data not shown).

The effects of chronic 14-day exposure on MSR were also analyzed using individual exposure durations (3, 7, 10 or 14 days of exposure) instead of repeated measurements to distinguish transient or delayed effects. While the effect of endosulfan is observed at every exposure duration, the potency of methylmercury increased with increasing exposure duration (LOEC) of methylmercury is an order of magnitude lower at exposure durations of 7 days or longer. On the other hand, the inhibition induced by α-cypermethrin appears transient as it is only observed at an exposure duration of 3 days. Effects of chlorpyrifos, chlorpyrifos-oxon and carbaryl were observed at exposure durations of 10 days or longer, although clear concentration-dependence could not be observed.

When representing the electrical activity as a percentage of electrodes on which substantial electrical activity (>0.1 spikes/s) is recorded, concentration-dependent inhibition of activity development is clearly observed in primary cortical cultures exposed to methylmercury (Fig. S2A). Inhibition is also observed in primary cortical cultures exposed to carbaryl, although concentration-dependence was less clear (Fig. S2D). No effects were observed using this representation of electrical activity in primary cortical cultures exposed to chlorpyrifos, chlorpyrifos-oxon, α-cypermethrin or endosulfan.

In comparative experiments, the effects of acute exposure to methylmercury or insecticides was determined in primary cortical cultures at DIV10. Acute exposure to methylmercury inhibited neuronal activity (represented as the treatment ratio of MSR) concentration-dependently (ANOVA $P < 0.0001$) to 41 ± 5% of control ($n = 19$) at a lowest observed effect concentration (LOEC) of 1 μM and complete inhibition (0.4 ± 0.2%, $n = 15$) at 10 μM (Fig. 3A). A comparable inhibitory effect was observed when exposing the cortical cultures acutely to chlorpyrifos, chlorpyrifos-oxon and α-cypermethrin with LOECs of 10, 10 and 1 μM, respectively (Fig. 3B, 3C, 3E and Table 1). Exposure to carbaryl inhibited spike rate in primary cortical cultures only at the highest concentration (ANOVA $P = 0.01$; 100 μM inhibited spike rate to 48 ± 6%, $n = 22$; $P < 0.05$ Student's *t* test; Fig. 3D). On the other hand, acute exposure to endosulfan concentration-dependently increased neuronal activity (ANOVA $P < 0.0001$) to 219 ± 21% of control ($n = 28$) at a LOEC of 0.3 μM and the maximum increase amounted to 288 ± 22% of control ($n = 33$) at 1 μM endosulfan (Fig. 3F). Noteworthy, exposure to 30 μM endosulfan largely inhibited spike rate, whereas activity ceased at 100 μM. Exposure to methylmercury, chlorpyrifos-oxon, carbaryl or endosulfan did not result in an effect on mISI. At their respective LOECs for MSR inhibition, a shift to higher mISI values is observed for chlorpyrifos and α-cypermethrin (data not shown).

When representing the electrical activity as a percentage of electrodes (during exposure compared to baseline) on which substantial electrical activity (>0.1 spikes/s) is recorded, acute methylmercury-, chlorpyrifos-, chlorpyrifos-oxon- and α-cypermethrin-induced inhibitions are also observed, although in all cases with similar or higher effect concentrations compared to when using the treatment ratio of the MSR (Fig. S3). Endosulfan, which clearly increased spike rate, did not affect the number of active electrodes except at the highest exposure concentrations at which spike rate was inhibited (Fig. S3F). Carbaryl did not affect the percentage of active electrodes (Fig. S3D).

3.3. Effects of exposure to methylmercury or insecticides on cell viability of primary rat cortical cultures

The observed effects on spontaneous electrical activity induced by chronic 14-day exposure were not associated with decreases in cell viability (Alamar Blue) measured subsequently to the last MEA recordings (at DIV21, i.e. after 14 days of exposure). Cell viability

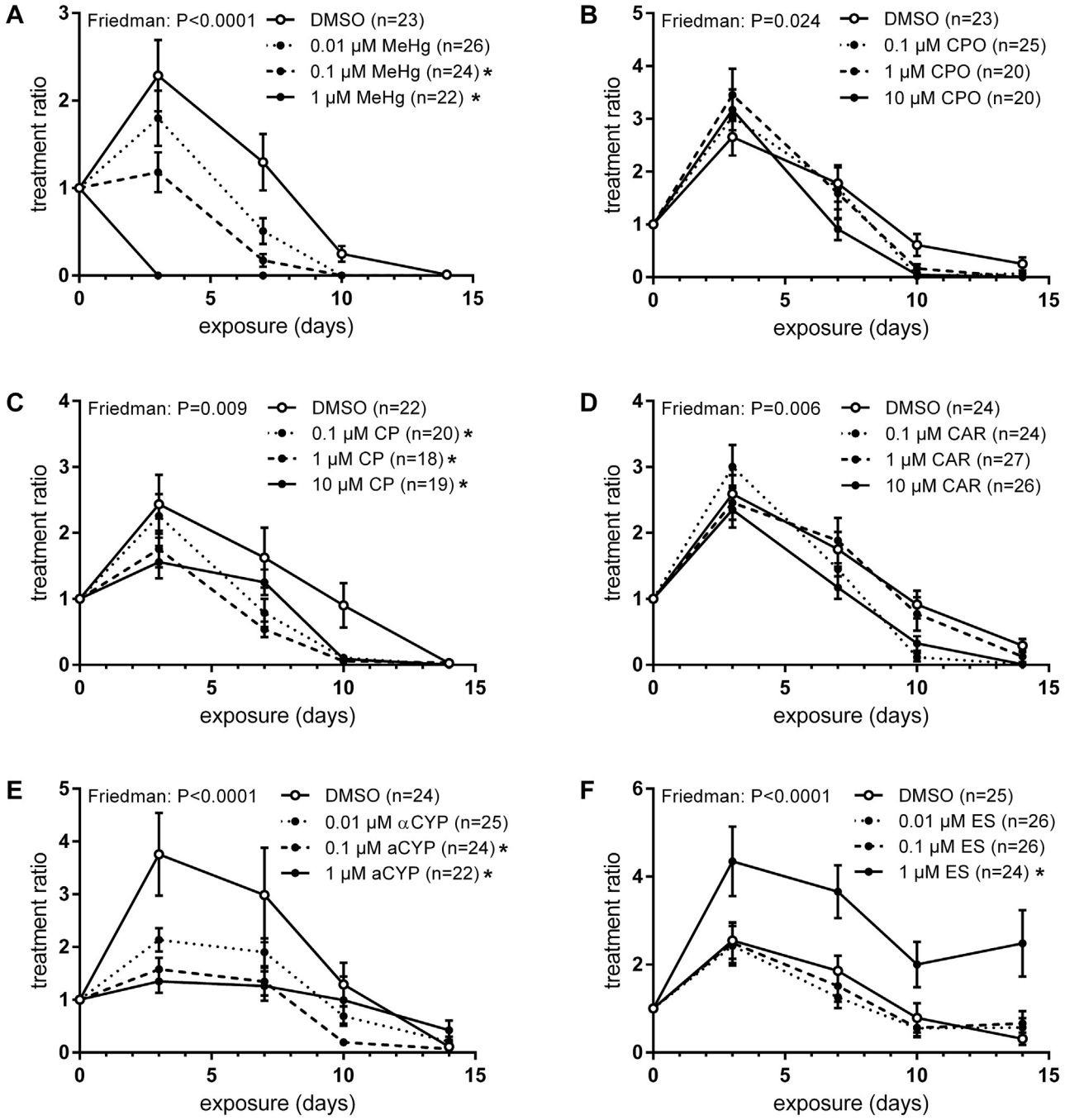

Fig. 2. Modulation of the development of spontaneous electrical activity in primary rat cortical cultures by chronic 14-day exposure to methylmercury (A) or different insecticides (B: chlorpyrifos-oxon; C: chlorpyrifos; D: carbaryl; E: α-cypermethrin; F: endosulfan). Effects on the development of electrical activity by chemical exposure are represented using the ratio of activity compared to the baseline activity recorded at DIV7 prior to exposure (in this graph: 0 on the X-axis). Effects on the development of spontaneous activity were tested using non-parametric Friedman tests (Friedman; P-values indicated within the graphs; n.s.: $P > 0.05$). The number of wells for each exposure conditions (n) are indicated within the graphs. In case of Friedman statistical significance, *post hoc* Bonferonni-corrected Wilcoxon signed-rank tests were used to identify which exposure conditions deviated from the solvent control (*significant deviation from control). Abbreviations: αCYP: α-cypermethrin; CAR: carbaryl; CP: chlorpyrifos; CPO: chlorpyrifos-oxon; ES: endosulfan; MeHg: methylmercury.

was not significantly affected in the cortical cultures exposed to either one of the insecticides (data not shown). A moderate decrease in cell viability was observed only in cortical cultures exposed to 1 μM methylmercury (to 62 ± 13% of control; n = 30).

The observed decreases in spontaneous electrical activity induced by acute exposure to chlorpyrifos-oxon, chlorpyrifos or α-cypermethrin were also not associated with decreases in cell viability measured in the MEA plates subsequently to the spontaneous electrical activity recordings. For methylmercury the acute inhibition of spontaneous electrical activity could be related to decreased cell viability only at the highest exposure concentrations (10 μM methylmercury reduced cell viability to 43 ± 5.2% of control; n = 18). Exposure to 30 μM methylmercury completely abolished cell viability, but at lower exposure concentrations (0.3–3 μM methylmercury), no effects of methylmercury on cell viability were observed (data not shown).

In parallel cultures, effects on cell viability in primary rat cortical cultures exposed for 24 h to insecticides or methylmercury

Table 1
Methylmercury and insecticides affect electrical activity at low exposure concentrations. Lowest observed effect concentrations (LOECs) of individual chemicals on the neuronal activity (spike rate) in primary cortical cultures. No effect concentrations (NOECs) are denoted between brackets.

	Chronic (14-day) exposure	Acute exposure
	LOEC (NOEC)[a]	LOEC (NOEC)[b]
methylmercury	0.1 μM (0.01 μM)	1 μM (0.3 μM)
chlorpyrifos-oxon	>10 μM (10 μM)	10 μM (3 μM)
chlorpyrifos	0.1 μM (<0.1 μM)	10 μM (3 μM)
carbaryl	>10 μM (10 μM)	100 μM (30 μM)[c]
α-cypermethrin	0.1 μM (0.01 μM)	1 μM (0.3 μM)
endosulfan	1 μM (0.1 μM)	0.3 μM (0.1 μM)

[a] Identified with Friedman test and *post hoc* Wilcoxon signed-rank tests.
[b] Identified with one-way ANOVAs and *post hoc* Bonferonni tests on logtransformed data.
[c] Identified with Student's *t* test.

were assessed by measuring effects on mitochondrial activity (AB assay) and membrane integrity (NR assay). These experiments also showed that the primary cortical cultures were most sensitive to exposure to methylmercury. Both mitochondrial activity and membrane integrity were concentration-dependently reduced by exposure to methylmercury, with a LOEC of 15 μM (Table 2). Such reductions were also observed for endosulfan, although only at the highest exposure concentration of 100 μM. Effects of the other investigated insecticides include moderate increases in mitochondrial activity (at concentrations ≥10 μM) and small decreases in membrane integrity (at concentrations ≥25 μM; Table 2).

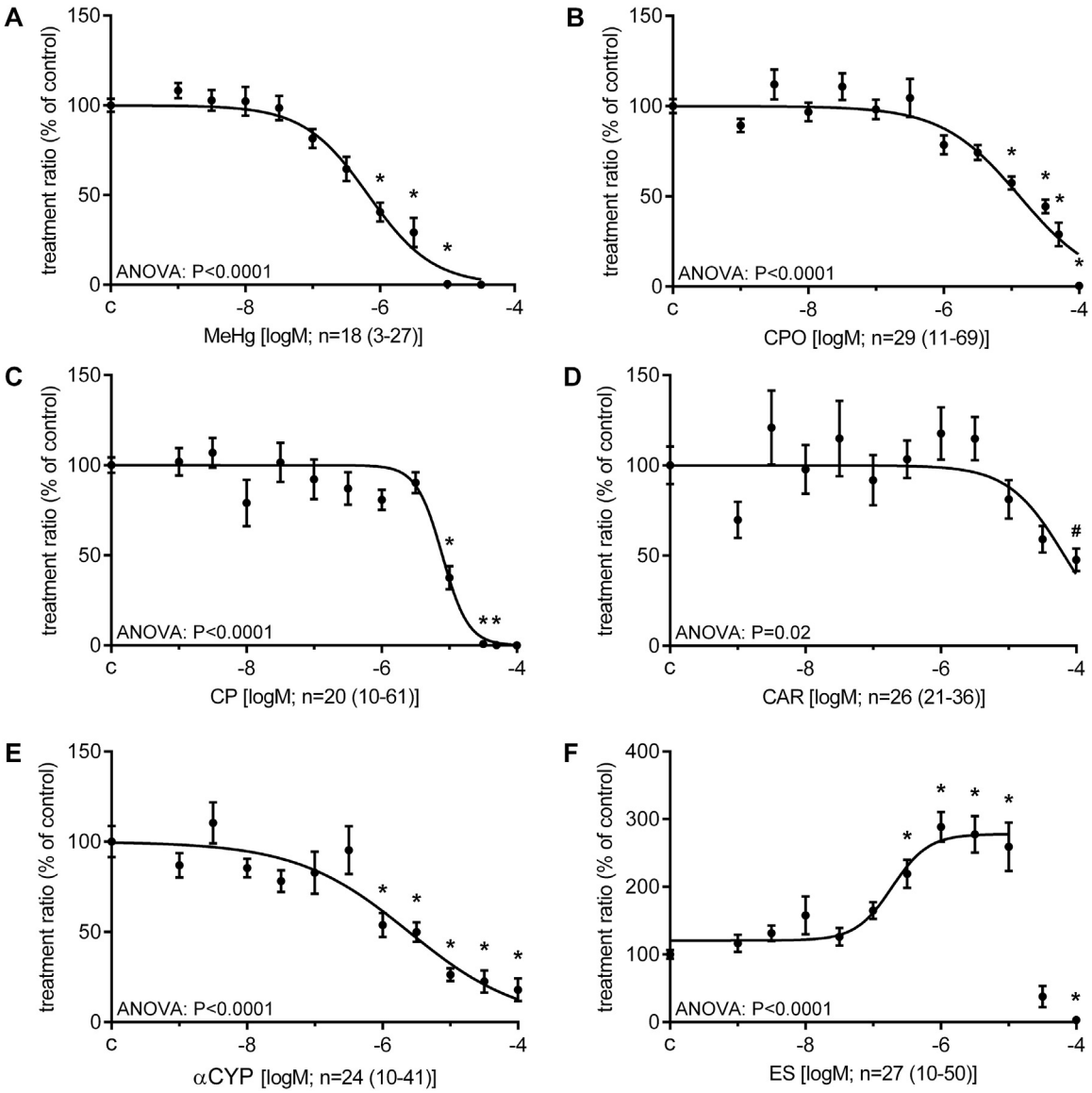

Fig. 3. Modulation of spontaneous electrical activity in primary rat cortical cultures by acute 30-min exposure to methylmercury (A) or different insecticides (B: chlorpyrifos-oxon; C: chlorpyrifos; D: carbaryl; E: α-cypermethrin; F: endosulfan). Relative change in activity was normalized to the solvent control (c) and data are presented here as average ± SEM with non-linear regression curves. Log-transformed data were used for statistical analysis. Average and range of the number of wells (n) for each exposure conditions and ANOVA P-values are indicated within the graphs (exact number of replicates per condition: Table S2). In case of ANOVA statistical significance, *post hoc* Bonferroni tests were used to identify which exposure conditions deviated from the solvent control (*significant deviation from control). For specific conditions, statistical significant difference from DMSO control was tested using Students *t*-test (# $P < 0.05$). Abbreviations: αCYP: α-cypermethrin; CAR: carbaryl; CP: chlorpyrifos; CPO: chlorpyrifos-oxon; ES: endosulfan; MeHg: methylmercury.

Table 2
Effects of 24 h exposure to methylmercury or different insecticides on measures of cell viability (parallel cultures in 24-wells plates). Effects were studied using the Alamar Blue (AB) assay for mitochondrial activity and the Neutral Red (NR) assay for membrane integrity. Values represent lowest observed effect concentrations (LOECs) on cell viability of primary cortical cultures from 3 individual isolations as identified with one-way ANOVAs and *post hoc* Bonferonni tests. Effect sizes at these LOECs (mean ± SEM) are denoted between brackets.

	LOEC AB assay	LOEC NR assay
methylmercury	15 μM (51 ± 7% of control; n = 18)	15 μM (40 ± 7% of control; n = 18)
chlorpyrifos-oxon	100 μM (148 ± 16% of control; n = 16)	25 μM (97 ± 1% of control; n = 18)
chlorpyrifos	10 μM (157 ± 19% of control; n = 17)[a]	>100 μM
carbaryl	>100 μM	100 μM (96 ± 1% of control; n = 17)
α-cypermethrin	50 μM (139 ± 8% of control; n = 16)[a]	>100 μM
endosulfan	100 μM (37 ± 3% of control; n = 17)	100 μM (56 ± 7% of control; n = 18)

[a] At a few higher exposure concentrations, effects were not statistically significant.

4. Discussion

Here we present the effects of chronic 14-day exposure to different insecticides or methylmercury on electrical activity in primary rat cortical cultures. Few *in vitro* neurotoxicity studies have included the investigation of effects of long-term exposures to these chemicals despite the fact that this is more realistic to the human exposure situation with regard to low-dose exposure.

Robust spontaneous activity was observed in control cultures during the second week of culture, after which the activity slowly ceased. Cell viability measurements demonstrated that cells are still viable during this later stage and neuronal activity could be increased by endosulfan and specific stimuli (not shown). Differences in the ontogeny of neuronal activity in primary cortical cultures of rat neonates have been observed between different labs (Figs. 1 and S1; Cotterill et al., 2016; Harrill et al., 2015). As for all types of primary cultures, there are several factors that can have an impact on variation between cortical cultures. On the one hand, there are inevitable differences in isolation procedures and culture conditions. On the other hand, there are differences associated with the litter and individual pups (e.g. exact developmental stage or litter size). These factors cause variation between cultures in the same lab, and therefore medium and solvent controls for concentration-effect relationships were always derived from the same isolation (litter) and measured in the same mwMEA plates in our study. Additionally, differences in data analysis can have an impact on profiles of activity development (Fig. S1). While in other instances only active electrodes are included in the data analysis, we have chosen to follow the activity development on all electrodes and wells on which a minimum baseline activity was recorded (at DIV7). A minimum activity criterion was thus not applied in subsequent recordings as decreased activity can be a result of exposure to a chemical. Consequently, this results in a lower average activity (Fig. S1), but it also allows for statistical approaches appropriate for repeated measurements as the number of electrodes and wells now remains constant over time. Moreover, inhibitory effects on network spike rate can be overlooked when only active electrodes are included in data analysis.

Chronic 14-day exposure to methylmercury, chlorpyrifos and α-cypermethrin inhibited the development of electrical activity, while exposure to endosulfan increased the activity. Chronic 14-day exposure to chlorpyrifos-oxon and carbaryl did not result in significant effects on neuronal activity. Effects of chronic 14-day exposure to methylmercury α-cypermethrin and endosulfan could be distinguished after 3 days of exposure, while effects of the other chemicals were not observed before exposure durations of at least 10 days. Effects on neuronal activity at LOEC were not confounded by decreased cell viability.

The effective concentration for the inhibition of neuronal activity was decreased by chronic 14-day exposure to methylmercury (LOEC: 1 μM) in comparison with the LOEC from the acute exposure study (LOEC: 0.3 μM). Such increased sensitivity in response to chronic 14-day exposure compared to acute exposure was to a limited degree also observed for insecticides. Lower effective concentrations compared to acute were observed from chronic 14-day exposure to low concentrations of chlorpyrifos and α-cypermethrin. For endosulfan, LOECs for effects of acute 30-min and chronic 14-day exposure are within the same order of magnitude. In the acute exposure setting, carbaryl has the highest LOEC, and a clear comparison with effect levels in the chronic 14-day exposure setting cannot be made due to the difference in concentration ranges used. Only for chlorpyrifos-oxon, the LOEC for effects of chronic 14-day exposure was higher compared to the acute LOEC possibly related to a relatively short half-life and/or only transient effects on electrical activity. From the comparison of LOECs in acute 30-min and chronic 14-day exposure settings it can be concluded that, although effective concentrations for effects of chronic 14-day exposure can be lower, acute concentration-response studies generally already reveal the potential for effects on neurophysiology resulting from prolonged chemical exposure. A similar conclusion with regard to the predictivity of the results induced by acute exposure compared to those induced by long-term exposure was recently drawn from a study investigating insecticide-induced effects on the calcium homeostasis by acute and subchronic 24-h exposure to insecticides (Meijer et al., 2014a, 2015).

Exposure of the cortical cultures was not started before activity was observed. Particular stages in neuronal development, which are attributed in particular to maturation of ion channel properties (Moody and Bosma, 2005), may thus already have passed to a certain degree. For developmental neurotoxicity testing, it needs further investigation whether exposure during the (very) early phase of network formation and maturation would evoke other or more potent effects from exposure to methylmercury or insecticides compared to those observed with the current chronic exposure paradigm.

Different types of parameters can be extracted from MEA recordings. In this study we mainly focused on effects on the spike rate (MSR). Other studies have presented MEA data also as a percentage of active electrodes (electrodes from which substantial activity is recorded). For comparability we have also used this parameter to present the effects of chemical exposure on electrical activity (percentage of active electrodes per well before and after exposure). It was observed in this study that this measure was comparable or less sensitive for most chronic 14-day and acute 30-min exposure conditions (Figs. S2 and S3). It was thus concluded that such analysis does not add critical information in this study. A similar conclusion can be drawn based on the few effects observed on ISI distribution, which are observed only at concentrations at which acute effect on spike rate are also observed. Although adding specific analyses and more parameters may contribute to the classification of chemicals based on their mechanism of action at concentrations affecting spike rate (Mack et al., 2014), several studies indicated that this does not reveal lower effective

concentrations (Defranchi et al., 2011; Shafer et al., 2008). Another study with a low number of replicates showed only a modest contribution by including other parameters of neuronal network activity (Alloisio et al., 2015).

In recent years there has been a number of other studies using primary cortical cultures grown on MEAs to investigate acute effects of chemicals on spike rate. These studies included methylmercury and most of the insecticides also included in the present research. In several previous studies, insecticides have been tested only at a single high concentration. This study thus contributes to the collection of neurotoxicity data from many different chemicals (Mundy et al., 2015), but also underlines the interlaboratory reproducibility of electrical activity data recorded from primary cortical cultures using MEA systems.

The inhibition of spike activity in primary cortical cultures on MEAs by carbaryl, chlorpyrifos-oxon, α-cypermethrin and methylmercury presented here are in line with in earlier studies (Alloisio et al., 2015; Defranchi et al., 2011; Johnstone et al., 2016; McConnell et al., 2012; Valdivia et al., 2014; Vassallo et al., 2016). However, in some of those studies, only the effects of a single high concentrations were tested (McConnell et al., 2012; Valdivia et al., 2014). Moreover, Alloisio et al. (2015) reported higher effect concentrations for chlorpyrifos compared to our study. Such differences may be related to interlaboratory differences in model characteristics, age of the cultures used for testing, (low) numbers of replicates, and differences in exposure paradigms (naïve culture for each replicate or cumulative dosing). Such interlaboratory variance is also demonstrated by Vassallo et al. (2016). Cumulative dosing, albeit efficient with regard to data collection, was avoided in our study to exclude the possibility that desensitization of ion channels and/or receptors after the first exposures obscures potential chemical-induced effects in subsequent exposure conditions (Zhang et al., 2013). The synchronization approach by Hondebrink et al. (2016) was followed to allow detection of possible transient effects during acute exposure (none detected in this study).

Full concentration-response curves in which increased activity is observed at low concentrations and strong inhibition at higher exposure concentrations demonstrate that there is a risk that such effects are not observed when only testing at high concentrations. Such biphasic effects were observed in our study for endosulfan, but have also been observed previously for other compounds. Exposure of primary cortical cultures on MEA to the insecticides fipronil or lindane, both also GABA receptor antagonists like endosulfan, resulted in an increased spike rate at low concentrations and inhibition at higher concentrations (Wallace et al., 2015). The inhibition by and effective concentration of α-cypermethrin observed in this current study is in accordance with the effects of a recent other study (Johnstone et al., 2016) as well as with effects of other pyrethroids (deltamethrin and permethrin) in earlier studies (Scelfo et al., 2012; Shafer et al., 2008; Vassallo et al., 2016). This is of particular relevance as co-exposure to different pyrethroids is common (Johnstone et al., 2016).

Primary cortical cultures form heterogeneous networks consisting of glial cells and excitatory and inhibitory neurons, with in particular glutamatergic and GABA-ergic properties (Hondebrink et al., 2016; Tukker et al., 2016; Weir et al., 2015). This model thus contains numerous potential molecular targets with which chemicals may interact. Such interactions with key structures and processes involved in the release or receival of inter- and intercellular signals within a neuronal network will result in changes in the electrical activity. As such, cortical cultures on mwMEAs provide an integrated measure to screen rapidly and reproducibly for effects on neurotransmission. In view of potential interspecies differences and 3R considerations, the use of human neuronal models for these types of studies is currently being explored (Tukker et al., 2016).

The observed changes could be the result of changes in one or more cellular and molecular processes involved in neurophysiology, such as loss of or reduced formation of connections, neuronal arborization or adaptive changes in ion channels, neurotransmitter receptors and gene expression. It can be further investigated within an ITS whether the observed effects on neuronal activity are due to such specific effects. Effects of the inhibition or activation/potentiation of a number of specific ion channels and pumps have already been recently demonstrated in the context of the evaluation of this system as an *in vitro* alternative for the mouse bioassay to detect marine biotoxins (Nicolas et al., 2014). Other studies showed that effects of chemicals on voltage-gated calcium and sodium channels and $GABA_A$ receptors could be demonstrated to be associated with effects observed in MEA studies with primary cortical cultures (Hondebrink et al., 2016; Valdivia et al., 2014). Combining these critical pieces of evidence from experimental studies and using this information in human risk assessment for (developmental) neurotoxicity will aid in the prevention of health effect resulting from exposure to environmental chemicals.

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Appendix A. Supplementary data

Supplementary data contains Tables S1 and S2, and Figs. S1–S3.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2016.10.002.

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