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Effects of acute insecticide exposure on neuronal activity *in vitro* in rat cortical cultures

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ABSTRACT

Exposure to pesticides, such as carbamates, organophosphates, organochlorines and pyrethroids, has been linked to various health problems, including neurotoxicity. Although most *in vivo* studies use only male rodents, some studies have shown *in vivo* sex-specific effects after acute exposure. Since *in vivo* studies are costly and require a large number of animals, *in vitro* assays that take sex-specific effects into account are urgently needed. We therefore assessed the acute effects of exposure to different carbamates (methomyl, aldicarb and carbaryl), organophosphates (chlorpyrifos (CPF), chlorpyrifos-oxon (CPO) and 3,5,6-trichloropyridinol), organochlorines (endosulfan, dieldrin and lindane) and pyrethroids (permethrin, alpha-cypermethrin and 3-phenoxy-benzoic acid (3-PBA)) on neuronal network function in sex-separated rat primary cortical cultures using micro-electrode array (MEA) recordings. Our results indicate that exposure to the carbamate carbaryl and the organophosphates CPF and CPO decreased neuronal activity, with CPO being the most potent. Notably, (network) burst patterns differed between CPF and CPO, with CPO inducing fewer, but more intense (network) bursts. Exposure to low micromolar levels of endosulfan induced a hyperexcitation, most likely due to the antagonistic effects on GABA receptors. Interestingly, females were more sensitive to endosulfan than males. Exposure to dieldrin and lindane also increased neuronal activity, albeit less than endosulfan and without sex-specific effects. Exposure to type I pyrethroid permethrin increased neuronal activity, while exposure to type II pyrethroid alpha-cypermethrin strongly decreased neuronal activity. The increase seen after permethrin exposure was more pronounced in males than in females. Together, these results show that acute exposure to different classes of pesticides exerts differential effects on neuronal activity. Moreover, it shows that MEA recordings are suited to detect sex-specific neurotoxic effects *in vitro*.

1. Introduction

During a lifetime people are exposed to numerous pesticides via food, water and air. There are many different classes of pesticides, including insecticides such as carbamates, organophosphates, organochlorines, and pyrethroids. Exposure to these insecticides has been linked to various health problems. Short-term exposure to insecticides can cause acute effects linked to the route of exposure, such as irritation of the eyes, skin or respiratory system. Additionally, short-term insecticide exposure is well-known to evoke systemic effects, including acute neurotoxicity (Flaskos, 2012). Long-term insecticide exposure may increase the risk of certain cancers, can cause reproductive issues and disrupts the endocrine system (Hazarika et al., 2019; Mnif et al., 2011; Mrema et al., 2013). Studies also associate chronic insecticide exposure with a higher prevalence of neurodegenerative diseases (Chin-Chan et al., 2015; Goldman, 2014; Sensi et al., 2017), and impaired neurodevelopmental outcomes (Flaskos, 2012; Grandjean and Landrigan, 2014; Lee et al., 2015a; Mrema et al., 2013).

Sex-specific differences following pesticide exposure are rather common. Some studies have shown *in vivo* sex-specific effects after acute insecticide exposure. High doses (>50 mg/kg) of chlorpyrifos decreased motor activity 1 day after exposure in both male and female adult rats. Interestingly, one week after the exposure these effects were still present in females, but not males (Mattsson et al., 1996). Other studies (González et al., 2021; Moser, 2000) also showed that neurobehavioral effects after acute organophosphate exposure can be sex-dependent. On

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the other hand, acute exposure to carbaryl (McDaniel et al., 2007; Ruppert et al., 1983), lindane (Rivera et al., 1998), permethrin and cypermethrin (Wolansky et al., 2006) decreased motor activity in male and female adult rats, with no sex-specific differences. Furthermore, studies show that acute exposure to insecticides can also influence behavior during adulthood, indicating that acute effects can cause long-lasting changes. Adult mice that were exposed to carbaryl, chlorpyrifos, endosulfan, or cypermethrin at postnatal day 10 showed differences in motor activity several months later (Lee et al., 2015a, 2015b). Although some sex-specific effects have been demonstrated *in vivo*, most studies focus on males and sex-specific effects have often not been addressed. Since *in vivo* studies are costly and require a large number of animals, there is an urgent need for an *in vitro* assay that can also take sex-specific effects into account.

One such *in vitro* assay is the multi-well microelectrode array (MEA) recording. A MEA consists of a cell culture surface with an integrated array of microelectrodes, which enables the recording of spontaneous neuronal activity. By measuring neuronal network activity, MEA recordings provide an integrated measure of possible effect(s) of chemical exposure on the underlying biochemical, morphological and electrophysiological endpoints. Neuronal networks grown on a MEA develop spontaneous activity over time and are responsive to a variety of drugs and chemicals, including pesticides (Kosnik et al., 2020; Strickland et al., 2018; van Melis et al., 2023). Furthermore, MEA recordings are considered to be relatively high-throughput and allow for non-invasive recording of neuronal activity (Johnstone et al., 2010; Mack et al., 2014). Primary rat cortical cultures, consisting mainly of excitatory glutamatergic and inhibitory GABAergic neurons (Hondebrink et al., 2016; Tukker et al., 2020), are the current standard for MEA recordings. Although research using MEAs has been done to determine the acute *in vitro* neurotoxicity of various pesticides, those studies focused on cell cultures from males only, or from 'mixed' cultures containing cells from both females and males, often in an unknown ratio. It is therefore unclear whether there are differences in acute *in vitro* neurotoxicity between males and females. Sex-separating primary rat cortical cultures makes it possible to use MEA recordings to investigate possible sex-specific effects *in vitro*.

The aim of the present study is to determine whether an *in vitro* approach, such as MEA recordings, can be used to investigate the effects of acute exposure to insecticides with different modes of action on spontaneous neuronal activity in a sex-specific manner. To this aim, we investigated the effects of several carbamates (aldicarb, carbaryl and methomyl), organophosphates (chlorpyrifos, chlorpyrifos-oxon and 3,5,6-trichloropyridinol (TCP)), organochlorines (endosulfan, dieldrin and lindane) and pyrethroids (permethrin, alpha-cypermethrin and 3-phenoxy-benzoic acid (3-PBA)) on neuronal activity in *in vitro* rat primary cortical cultures.

2. Methods

2.1. Chemicals

Chlorpyrifos oxon (purity 98.7 %) was obtained from AccuStandard (New Haven, CT, USA). Aldicarb (purity 99.9 %), carbaryl (purity 99.7 %) and DDE (purity 99 %) were obtained from Riedel-de Haën (Seelze, Germany). 3-phenoxy-benzoic acid (3-PBA; purity 99.7 %) was obtained from Dr. Ehrenstorfer (Augsburg, Germany). Phenol-red free neurobasal-A (NB-A) medium, L-glutamine (200 mM), glutamate (3.5 mM) penicillin/streptomycin (5000 U/mL/5000 mg/mL) and B-27 plus supplement were purchased from Life Technologies (Bleiswijk, the Netherlands). Unless otherwise noted, all other chemicals were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). Pesticide stock solutions of 10^{-1} M to 10^{-5} M were prepared in dimethyl sulfoxide (DMSO) and diluted in culture medium just prior to the experiments. All solutions used in experiments, including control experiments, contained 0.1 % DMSO.

2.2. Cell culture

Sex-specific, primary cultures of rat cortical neurons were prepared from pups born of timed-pregnant Wistar rats (Envigo, Horst, the Netherlands) on postnatal day 0 or 1 as described previously (Gerber et al., 2021). Briefly, rat pups were sexed by looking at the anogenital distance and separated between males and females. Next, pups were decapitated and the cortices were isolated and placed in ice-cold dissection medium (450 mL NBA medium, 14 g sucrose, 1.25 mL L-glutamine (200 mM), 5 mL glutamate (3.5 mM), 5 mL penicillin/streptomycin and 10 mL B-27, pH 7.4). Cortices were minced and triturated to a homogenous suspension and filtered through an easy strainer (100 μm, Greiner Bio One, Alphen aan den Rijn, The Netherlands). Subsequently, cells were centrifuged for 5 min at 800 rpm. The supernatant was removed and the pellet was resuspended using 1 mL of dissection medium per rat brain and diluted to a cell suspension containing 2×10^6 cells/mL. Next, drops (50 μL/well) of cell-suspension were seeded at a density of 1×10^5 cells/well on PEI (0.1 % PEI solution in borate buffer (24 mM sodium borate / 50 mM boric acid in Milli-Q adjusted to pH 8.4)) coated 48-well microelectrode array (MEA) plates (Axion Biosystems Inc., Atlanta, GA, USA). Cells were allowed to attach in a humidified 5 % CO_2/95 % air atmosphere for 2 hours at 37 °C, before 450 μL dissection medium was added to each well. At four days *in vitro* (DIV 4), 450 μL dissection medium was replaced by 450 μL glutamate-free medium (450 mL NBA medium, 14 g sucrose, 1.25 mL L-glutamine (200 mM), 5 mL penicillin/streptomycin and 10 mL B-27, pH 7.4). Cells were cultured in 5 % CO_2/95 % air atmosphere at 37 °C until use at DIV 9–11.

All animal experiments were performed in agreement with Dutch law, the European Community directives regulating animal research (2010/63/EU) and approved by the Ethical Committee for Animal Experiments of Utrecht University. All efforts were made to minimize the number of animals used and their suffering.

2.3. MEA recordings

Multi-well MEA plates were used to record spontaneous neuronal activity. These plates contain 48 wells per plate, each well containing an array of 16 individual embedded nanotextured microelectrodes (40–50 μm diameter; 350 μm center-to-center spacing), yielding a total of 768 channels, which can be recorded at the same time. Recordings were made as previously described (Gerber et al., 2021). All compounds were tested at final concentrations of 0.01–100 μM, except for permethrin for which solubility limitations prevented testing > 30 μM. Each well was exposed to only one condition (i.e., one concentration of one test compound) to prevent potential effects of cumulative dosing.

On DIV 9–11, a 48-well MEA plate was placed in a Maestro 768-channel amplifier with integrated heating system (set at 37 °C), temperature controller and data acquisition interface (Axion Biosystems Inc., Atlanta, GA, USA). Prior to each recording, MEA plates were allowed to equilibrate for around 5 minutes, after which a 30 minute baseline recording of spontaneous activity was started. Wells with at least four bursting electrodes at baseline recording were included for experiments. After the baseline recording, a 35 minute exposure recording was started to determine the acute effects of the test compounds on spontaneous neuronal activity (spiking and bursting behavior). During the first 5 minutes of this recording, all wells were exposed individually by manually pipetting 55.5 μL of different concentrations of test compounds or vehicle (DMSO control) to each active well.

2.4. Cell viability assay

Effects of test compounds on cell viability were assessed using the Alamar Blue (AB) assay (protocol adapted from Bopp and Lettieri, 2007). Cells were exposed to the highest concentration (30 μM for permethrin; 100 μM for all other compounds) for 24 h. If effects were

found at this concentration, lower concentrations were tested. Mitochondrial activity of the cells was recorded as a measure of cell viability with the AB assay, which is based on the ability of the cells to reduce resazurin to resorufin. Briefly, following exposure, cells were incubated for 75 min with 12,5 μM AB solution in HBSS (Invitrogen, Breda, The Netherlands). Resorufin was measured spectrophotometrically at 540/590 nm (Infinite M200 microplate; Tecan Trading AG, Männedorf, Switzerland).

2.5. Data analysis and statistics

Data analysis for the MEA data was performed as described in Gerber et al. (2021). Briefly, MEA data acquisition was managed with Axion's Integrated Studio (AxIS version 2.6). Raw data files were obtained by sampling channels simultaneously with a gain of 1200x and a sampling frequency of 12.5 kHz/channel using a band-pass filter (200–5000 Hz).

Raw data were pre-processed to obtain.spk files. Spikes were detected using the AxIS spike detector (Adaptive threshold crossing, Ada BandFlt v2) with a post/pre-spike duration of 3.6/2.4 ms and a spike threshold of 7 × SD of the internal noise level (rms) of each individual electrode. Spike information was then further analysed using NeuralMetrics Tool (v3.1.7, Axion BioSystems) and custom-made macros in Excel. Electrodes that recorded ≥ 6 spikes per minute were included in the analysis. A burst is a cluster of spikes measured on a single electrode. Bursts were defined using the Poisson surprise method (Legendy and Salcman, 1985) with a minimum of 10 surprises. A network burst is a coordinated cluster of spiking across multiple electrodes. Network bursts were defined using an adaptive threshold with a minimum of 40 spikes, each separated by a maximum interval set automatically on a well-by-well basis based on the mean spike rate of each well, for a minimum of 15 % of the electrodes/well. If we did not observe transient effects, data from the last 10 minutes of the 30 minute exposure recording were used for analysis, since this is the most stable timeframe (see (Hondebrink et al., 2016)). When transient effects were found, the raster plots for the total 30 minutes exposure time were examined to determine when these transient effects took place.

Based on literature and a principal component analysis (PCA), the ten most important MEA parameters were determined (see Supplementary data, Table 1). These are number of spikes, number of (network) bursts, inter-burst interval, (network) burst duration, mean inter-spike interval (ISI) within network bursts, number of spikes per (network) burst, and area under cross-correlation, a measure for synchronicity. The PCA was conducted in R using the packages FactoMineR (by Husson et al., 2022; version 2.7) and FactoExtra (by Kassambara & Mundt, 2020; version 1.0.7) as described in (Tukker et al., 2020) with minor modifications. First, highly correlated parameters were determined per compound based on visual inspection of the heatmap and subsequently removed from the data set. Next, a scree plot was created for each compound to visualize the percentage of variation explained by each component. Based on the PCA, the percentage of concentration-dependent variation explained by each parameter was calculated for all compounds. Parameters that did not explain variation were excluded. Parameters contributing more than the expected contribution of 7 % (expected value = (1/number of variables * 100 %)) were considered important for a component.

For each experimental condition, primary cultures originating from at least three different isolations were used. The data represent average values derived from 15 to 34 wells (*n*) from 3 to 8 (N) independent experiments and are presented as mean % ± standard error of the mean (SEM) compared to solvent control (treatment ratio). Experimental values that exceeded mean ± 2x SD (of their respective condition) were considered to be outliers (3,8 % outliers) and therefore excluded from further analysis. Descriptive statistics (coefficient of variation (% CV), number of plates (*N*), and number of wells (*n*)) for each measured endpoint can be found in table S2 (carbamates), table S3 (organophosphates), table S4 (organochlorines) and table S5 (pyrethroids). Benchmark response (BMR) cut-offs were based on the average variation in all pooled DMSO control experiments. Effects that are smaller than the BMR are considered to be of limited toxicological relevance, even if significantly different from control (indicated with asterisks). Statistical analyses were performed using GraphPad Prism v9.2.0 (GraphPad Software, San Diego, CA, USA) using one-way ANOVA (cell viability and basal male-female differences) and two-way (two factors; concentration and sex) ANOVA with Tukey post-hoc tests. A p-value ≤ 0.05 was considered statistically significant. Concentration-response curves were fitted using a nonlinear sigmoidal or bell-shaped curve-fit when applicable.

3. Results

To verify if any sex-specific effects found here are not due to differences in baseline neuronal activity, we compared the mean spike, burst and network burst frequency, (network) burst duration, number of spikes per (network) burst, mean ISI within network bursts and synchronicity in all wells for males (n = 1976) and females (n = 1928). Males and females only differed in the number of spikes per network burst (higher in males), with all other parameters showing no differences (Table 1).

3.1. Carbamates

Carbaryl showed effects only at the highest concentration (100 μM), with in both sexes a decrease in the number of spikes, bursts, number of spikes per burst and synchronicity, but an increase in inter-burst interval. Males, but not females, showed a decrease in the number of network bursts, while females showed a decrease in the number of spikes per network burst and an increase in mean ISI within network bursts that was not seen in male cultures. The increase in inter-burst interval was more pronounced in males than in females (Fig. 1, Fig. S1A and Fig. S2). Exposure to aldicarb and methomyl did not affect neuronal activity up to 100 μM (Fig. 1 and Fig. S1B-C). The effects found here were not due to cytotoxicity (Fig. S3).

Altogether, the carbamates tested here have little to no effect on neuronal activity except for carbaryl at 100 μM.

3.2. Organophosphates

Both acute exposure to chlorpyrifos (CPF) and its metabolite chlorpyrifos-oxon (CPO) concentration-dependently inhibited the number of spikes, bursts, network bursts and synchronicity (Fig. 2 and Fig. S4A-B). For the number of spikes, bursts and network bursts, CPO proved to be more potent, while the reverse was true for synchronicity. After exposure to 100 μM CPF, (network) burst duration was decreased and inter-burst interval was increased (Fig. 2 and Fig. S4A). The number of spikes per (network) burst was more sensitive, with inhibition occurring after exposure to 10 μM CPF. Exposure to 10 μM CPF

Table 1
Average baseline neuronal activity ± SD per well in male and female cultures.

	Male	Female
Spike frequency (Hz)	24.12 ± 11.9	22.20 ± 11.3
Burst frequency (Hz)	1.15 ± 0.65	1.11 ± 0.57
Burst duration (seconds)	0.18 ± 0.17	0.19 ± 0.41
Spikes per burst	15.93 ± 6.52	15.21 ± 6.49
Inter-burst interval (seconds)	25.2 ± 18.97	25.98 ± 18.35
Network burst frequency (Hz)	0.13 ± 0.07	0.13 ± 0.07
Network burst duration (seconds)	0.38 ± 0.28	0.36 ± 0.27
Spikes per network burst	200 ± 131 **	182 ± 123 **
Mean ISI within network burst (seconds)	0.0023 ± 0.0018	0.0024 ± 0.0018
Synchronicity (area under cross-correlation)	6.06 ± 5.32	5.13 ± 4.53

** indicates differences between male and female cultures (p < 0.01).

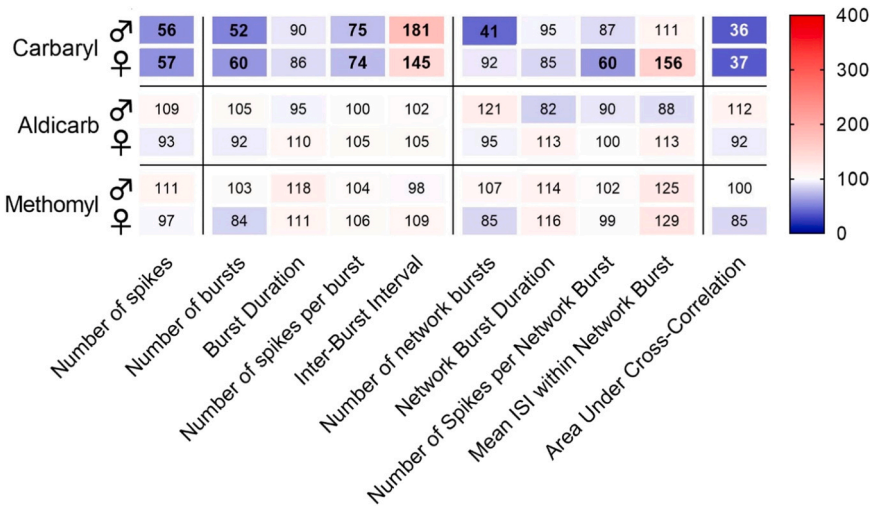

Fig. 1. Heatmap with a double gradient (red is increase; blue is decrease) showing the acute effects of exposure of male and female primary rat cortical cells for 30 minutes to 100 μM carbaryl (top), aldicarb (middle) and methomyl (bottom) on selected neuronal activity parameters. Mean treatment ratios are depicted in percentage of DMSO control for each parameter. Concentrations of < 100 μM were without effect. Values depicted in bold differ significantly ($p<0.05$) from DMSO control.

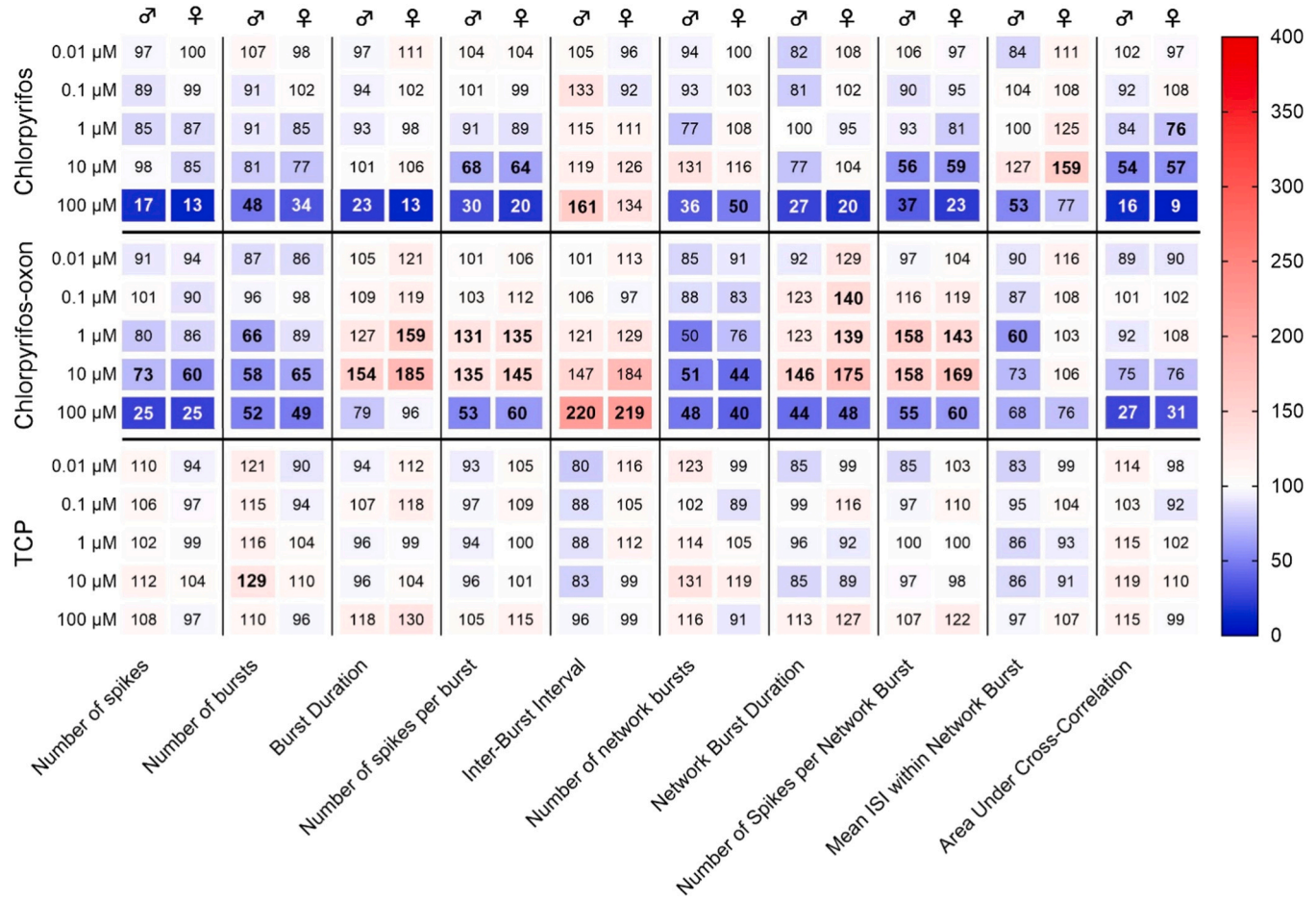

Fig. 2. Heatmap with a double gradient (red is increase; blue is decrease) showing the acute effects of exposure of male and female primary rat cortical cells for 30 minutes to chlorpyrifos, chlorpyrifos-oxon and 3,5,6-trichloropyridinol (TCP) on selected neuronal activity parameters. Mean treatment ratios are depicted in numbers in percentage of DMSO control for each parameter. Values depicted in bold differ significantly ($p<0.05$) from DMSO control.

increased the mean ISI within network bursts in both sexes, but this increase was only significant in females (Fig. 2 and Fig. S4A). After exposure to CPO, (network) burst duration and the number of spikes per (network) burst showed a reverse U-shape effect, with increases in (network) burst duration and the number of spikes per (network) burst at 1 and 10 μM, but decreases at 100 μM (Fig. 2 and Fig. S4B).

Interestingly, the increase in (network) burst duration was more pronounced in females than in males. Inter-burst interval was increased in both sexes after acute exposure to 100 μM CPO (Fig. 2 and Fig. S4B), reflecting the decrease in the number of bursts. No clear effects were found on mean ISI within network bursts. Acute exposure to the hydrolyzed CPF metabolite TCP did not affect any of the parameters (Fig. 2 and Fig. S4C).

Concluding, both CPF and its bioactivated metabolite CPO inhibit neuronal activity, but the (network) burst patterns differ between the two compounds (Fig. S5). Exposure to 1 and 10 μM CPO decreases the number of (network) bursts, but increases their intensity, with longer (network) burst durations and more spikes per (network) burst (Fig. S5B). Exposure to CPF, however, has no effect on the number of (network) bursts and (network) burst duration at concentrations < 100 μM, while it reduces the number of spikes per (network) burst at 10 μM (Fig. S5A). The detoxified metabolite TCP does not affect neuronal activity. The effects found after exposure to CPF and CPO were not due to cytotoxicity (Fig. S3).

3.3. Organochlorines

Acute exposure to endosulfan resulted in an inverted U-shape for the number of spikes, bursts, network bursts and synchronicity, with increases at 0.1–10 μM and a strong inhibition at 100 μM (Fig. 3 and Fig. 4). Interestingly, the excitatory effects were more pronounced in females than in males (Fig. 3 and Fig. S6). No effects were found on burst duration, but network burst duration was decreased at 1 and 10 μM (Fig. 4 and Fig. S7). The number of spikes per (network) burst was increased after exposure to 1 and 10 μM (Fig. 4 and Fig. S7). Inter-burst interval and mean ISI within network bursts were decreased from 0.1 μM to 10 μM (Fig. 4 and Fig. S7). Because of the complete inhibition of activity at 100 μM, (network) burst duration, number of spikes per (network) burst, inter-burst interval and mean ISI within network bursts could not be determined for this concentration.

Acute exposure to lindane also resulted in an inverted U-shape for the number of spikes, burst and synchronicity, but no effects were found on the number of network bursts (Fig. 4, Fig. S8A and Fig. S9). In contrast to endosulfan exposure, there were no sex-specific differences in these four parameters. (Network) burst duration was increased after exposure to 10 μM, but only reached significance in females. The number of spikes per (network) burst increased at 1 and 10 μM, with effects being more pronounced in females. The number of spikes per burst, but not network bursts, were slightly decreased after 100 μM exposure. Inter-burst interval was reduced after 1 and 10 μM exposure, mirroring the increase in the number of bursts. Mean ISI within network bursts was inhibited after 1 and 10 μM, which can be attributed to the increase in the number of spikes per network burst.

For dieldrin, transient exposure effects were found (see Fig. S10 for an example) and thus different exposure windows were analyzed. Analysis for the first 10 minutes after exposure to dieldrin showed an increase in the number of spikes and synchronicity (Fig. 4 and Fig. S8B). Inter-burst interval decreased after >1 μM exposure to dieldrin. In males, but not females, the number of bursts was increased after >1 μM exposure, while the number of network bursts was slightly decreased at 100 μM. Burst duration was increased at 100 μM, while network burst duration was not affected. Number of spikes per (network) burst increased in both sexes after exposure to 1, (only for network burst), 10 and 100 μM. Mean ISI within network bursts was decreased after >1 μM exposure to dieldrin.

Analysis in the time-window 20 minutes after exposure to dieldrin resulted in an inverted U-shape for the number of spikes and synchronicity in both sexes (Fig. 4 and Fig. S8C). In males, but not females, the number of bursts was increased after 1 μM exposure, while the number of network bursts was slightly decreased at > 10 μM. Burst duration was

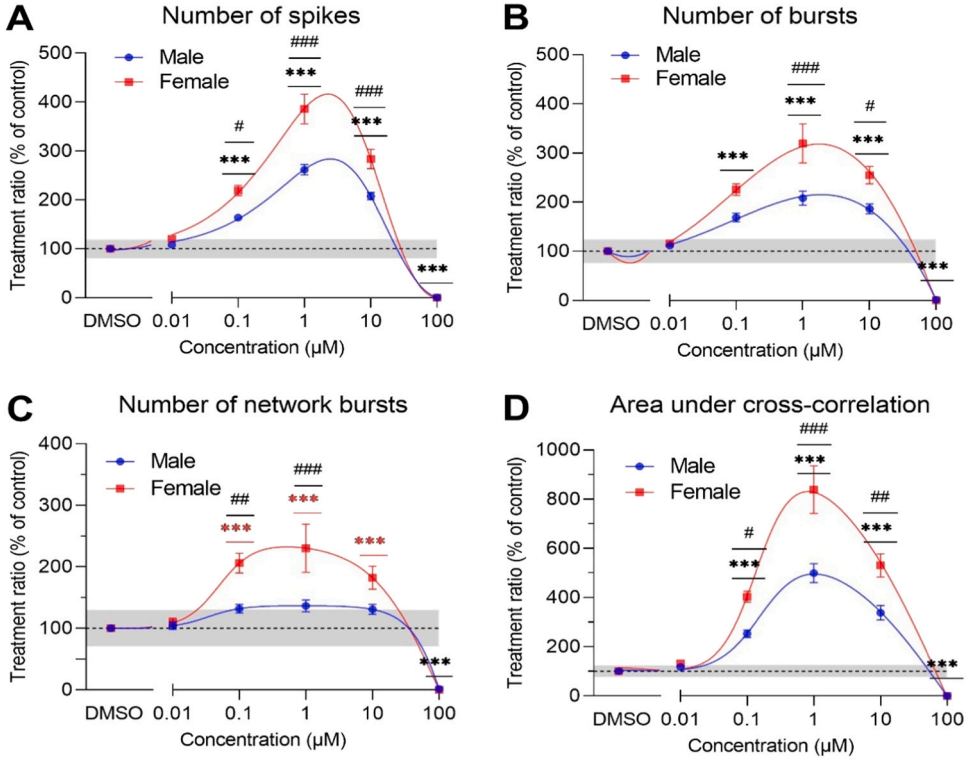

Fig. 3. Sex-specific effect of 30 minute acute exposure to endosulfan on number of spikes (A), bursts (B), network bursts (C) and synchronicity (D) parameters. The grey shaded area represents a benchmark response derived from the average variation in DMSO control experiments. Data points display average percentage compared to control (DMSO control set to 100%) ± SEM from 23 to 28 individual cells (≥ 7 independent experiments per concentration). Difference from DMSO control (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). Color of asterisks indicates which parameter is significantly affected (blue for males; red for females; black if both sexes differ significantly from solvent control). Difference between sexes (#$p \leq 0.05$; ## $p \leq 0.01$; ### $p \leq 0.001$).

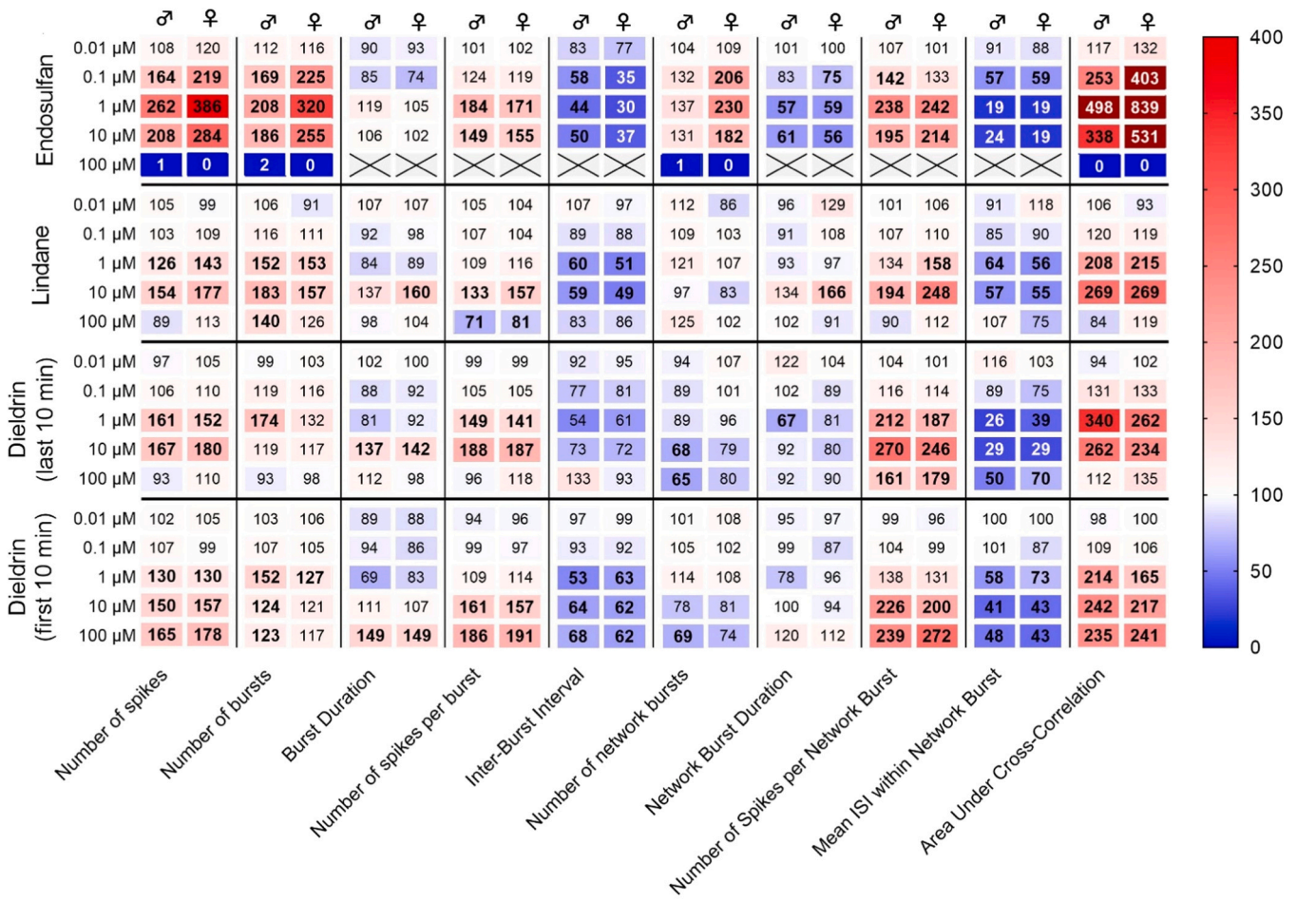

Fig. 4. Heatmap with a double gradient (red is increase; blue is decrease) showing the acute effects of exposure of male and female primary rat cortical cells for 30 minutes to endosulfan, lindane, and dieldrin, on selected neuronal activity parameters. For dieldrin, the effects found in the first 10 minutes after exposure and after 20 minutes of exposure are shown. Mean treatment ratios are depicted in numbers in percentage of DMSO control for each parameter. Values depicted in bold differ significantly ($p<0.05$) from DMSO control.

increased at 10 µM, but no clear effects were found on network burst duration. The number of spikes per (network) burst increased in both sexes after exposure to 1, 10 and 100 (only for network bursts) µM. The latter was reflected in the mean ISI within network bursts, which was decreased after >1 µM exposure to dieldrin. No significant effects were found on inter-burst interval.

Concluding, endosulfan, lindane and dieldrin stimulate neuronal activity at sub- and low micromolar concentrations, but inhibit neuronal activity at high micromolar concentrations. Endosulfan was the most potent of these three organochlorines. Moreover, after endosulfan exposure we found a sex-specific effect, with the excitatory effects being more pronounced in females. Exposure to 100 µM endosulfan results in overt cytotoxicity (Fig. S3), but concentrations ≤10 µM that evoked a clear hyperexcitation did not affect cell viability (data not shown). The effects found after exposure to dieldrin and lindane were not due to cytotoxicity (Fig. S3).

3.4. Pyrethroids

Acute exposure to permethrin concentration-dependently increased the number of spikes in both sexes, with a stronger effect in males (Fig. 5, Fig. S11A and Fig. S12). For the number of bursts, network bursts, and synchronicity, this increase was only significant in males (Fig. S11A). There were no effects on (network) burst duration and the number of spikes per (network) burst in both sexes. Inter-burst interval was not affected either, despite the significant increase in the number of bursts in males. Mean ISI was increased in males after exposure to 30 µM permethrin (Fig. S11A).

At ≥1 µM, alpha-cypermethrin exposure resulted in a strong concentration-dependent decrease in the number of spikes, bursts, network bursts and synchronicity in both sexes (Fig. 5 and Fig. S11B). Network burst duration was increased after 1 µM exposure, while the number of spikes per network burst slightly decreased. There were no effects on burst duration and the number of spikes per burst at this concentration. Inter-burst interval showed a small increase after exposure to 1 µM (only significant in females; Fig. S11B). Mean ISI within network bursts was significantly increased for both sexes after exposure to 1 µM alpha-cypermethrin (Fig. S11B). Because of the (almost) full inhibition of activity at 10 and 100 µM, (network) burst duration, number of spikes per (network) burst, inter-burst interval and mean ISI within network bursts could not be determined for these concentrations. No clear effects of acute exposure to 3-PBA were found (Fig. 5 and Fig. S11C).

Concluding, permethrin stimulates neuronal activity at a high concentration, with a more pronounced effect in males. Alpha-cypermethrin, however, strongly decreases neuronal activity. Exposure to the pyrethroid metabolite 3-PBA has no effect on neuronal activity. The effects found after exposure to permethrin and alpha-cypermethrin were not due to cytotoxicity (Fig. S3).

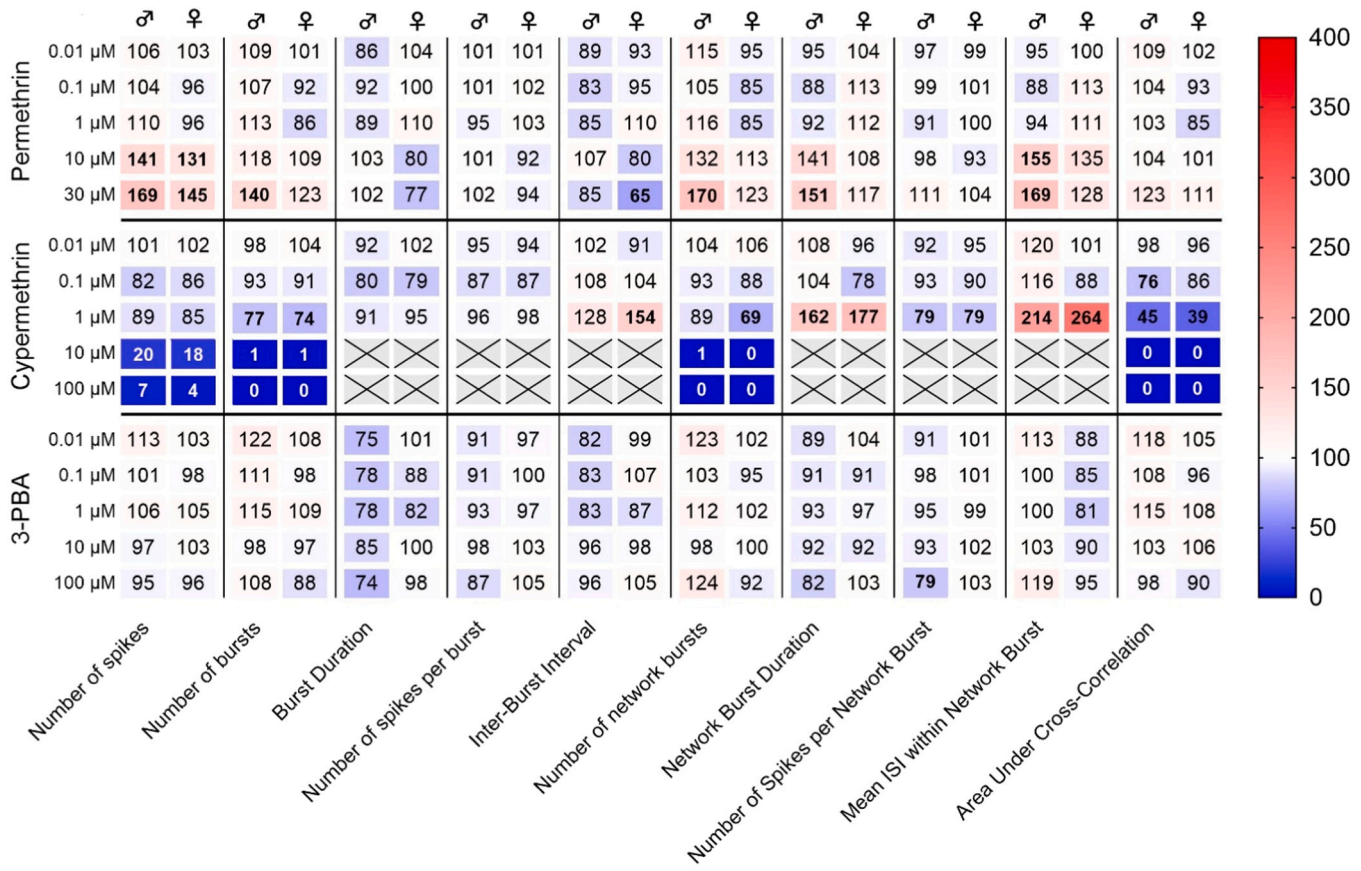

Fig. 5. Heatmap with a double gradient (red is increase; blue is decrease) showing the acute effects of exposure of male and female primary rat cortical cells for 30 minutes to permethrin, alpha-cypermethrin and 3-phenoxybenzoic acid (3-PBA) on selected neuronal activity parameters. Mean treatment ratios are depicted in numbers in percentage of DMSO control for each parameter. Values depicted in bold differ significantly ($p<0.05$) from DMSO control.

4. Discussion

Our study aimed to determine whether an *in vitro* approach can be used to investigate the effects of acute exposure to insecticides in a sex-specific manner. To this aim, we used micro-electrode array (MEA) recordings, which enabled us to measure neuronal activity before and after exposure to chemicals in a non-invasive way. We found concentration-dependent effects on neuronal activity after acute exposure to the different insecticides. Since these insecticides had different modes of action, the direction and degree of effects were compound-specific. Interestingly, for some compounds these effects differed between male and female rat cortical cultures, confirming that the MEA can be used as an *in vitro* approach to study sex-specific effects. While some earlier studies included sex-specific effects in vitro following chronic or developmental exposure, to our knowledge there are only a few studies covering sex-specific effects in vitro after acute exposure (Dietrich et al., 2001 on the effects of ochratoxin A and B in kidney; Mennecozzi et al., 2015 on the effects of hepatotoxic drugs in liver), with only Astiz et al., (2014) focusing on neurotoxicity. This further highlights the novelty of our approach and the results presented here.

Carbamates and organophosphates exert their acute neurotoxicity mainly through inhibition of acetylcholinesterase (AChE; see Table 2). This inhibition leads to an accumulation of acetylcholine in the synaptic cleft and transient overstimulation of acetylcholine receptors, followed by receptor desensitization and paralysis (Bas et al., 2019; Richardson et al., 2019). The inhibition of AChE is irreversible for organophosphates, while AChE inhibition by carbamates is reversible and lasts only minutes to hours (McDaniel et al., 2007; Moser et al., 2015; Risher et al., 1987).

Exposure to 100 µM carbaryl decreased neuronal activity, while exposure to aldicarb and methomyl had no effects at the tested concentrations. This is in line with earlier MEA findings in which exposure to 40 µM carbaryl decreased neuronal activity, while exposure to the

Table 2
List of compounds studied here with their main mechanisms of action (MoA), their effect on overall neuronal activity, and the *in vitro* lowest effect concentration (LOEC) found on the MEA. AChE: acetylcholinesterase. VGSC: voltage-gated sodium channel.

Compound	Main MoA	Effect on MEA	*In vitro* LOEC
Aldicarb	Inhibition of AChE (transient)	No effect	N/A
Methomyl	Inhibition of AChE (transient)	No effect	N/A
Carbaryl	Inhibition of AChE (transient)	Decrease	100 µM
Chlorpyrifos	Inhibition of AChE	Decrease	10 µM
Chlorpyrifos-oxon	Inhibition of AChE	Decrease	1 µM
TCP	-	No effect	N/A
Endosulfan	$GABA_A$-receptor antagonist	Reversed U-shape	0.1 µM
Dieldrin	$GABA_A$-receptor antagonist	Reversed U-shape	1 µM
Lindane	$GABA_A$-receptor antagonist	Reversed U-shape	1 µM
Permethrin	Prolonged open-time of VGSC	Increase	10 µM
α-Cypermethrin	Prolonged open-time of VGSC	Decrease	1 µM
3-PBA	-	No effect	N/A

same concentration of aldicarb did not affect neuronal activity (Valdivia et al., 2014). Moreover, the difference between carbaryl and other carbamates is also seen *in vivo*, where methomyl did not affect behavior in rats, while carbaryl decreased motor activity (McDaniel et al., 2007). Interestingly, number of network bursts was decreased in males, but not females, after exposure to 100 μM carbaryl. The opposite was true for the number of spikes per network bursts, which was decreased in females, but not in males. These findings can be explained by the settings that were used in our study to detect network bursts (40 spikes per network burst). When lowering the number of spikes necessary to be counted as network burst, the sex-specific effect on both number of network bursts and number of spikes per network burst disappeared. The limited decrease in neuronal activity after carbamate exposure is in line with an earlier study (Meijer, Hamers, et al., 2014), which showed that carbamates had only limited effects on voltage-gated calcium channels (VGCCs). It cannot be excluded that the inhibitory effect seen here at 100 μM is due to the disturbance of VGCCs, as the highest tested concentration of carbaryl in (Meijer, Hamers, et al., 2014) was only 10 μM.

The tested organophosphates inhibited neuronal activity at lower concentrations than carbamates, and thus proved to be more potent inhibitors of neuronal activity. Neuronal activity decreased after exposure to CPF (> 10 μM) and CPO (> 1 μM), with rat cortical cells being more sensitive to CPO on multiple parameters of neuronal activity. These results are in line with earlier MEA work that showed inhibition of neuronal activity after exposure to 40 μM CPF and CPO (Valdivia et al., 2014). We previously showed that MEA recordings are not very sensitive to AChE inhibition (van Melis et al., 2023), most likely due to the low number of cholinergic neurons and thus very low ACh levels. Therefore, we conclude that the decrease in neuronal activity after exposure to carbaryl, CPF and CPO is most likely not due to AChE inhibition. The effects found here are in line with earlier studies that indicated that CPF and CPO can reduce depolarization-evoked Ca^{2+} influx and neuronal activity (Dingemans et al., 2016; van Melis et al., 2023).

Exposure to the organochlorines endosulfan, dieldrin and lindane resulted in a hyperexcitation at low micromolar concentrations, followed by an inhibition after exposure to higher concentrations. The increase in activity found here is in line with earlier MEA findings (Mack et al., 2014; Saavedra et al., 2021; Valdivia et al., 2014) and is most likely due to the antagonistic effect of these organochlorines on $GABA_A$-receptors (Croom et al., 2015; Jang et al., 2016). The decrease in activity after exposure to higher concentrations is likely caused by inhibitory effects on VGCCs (Heusinkveld et al., 2010; Meijer, Dingemans, et al., 2014). For dieldrin, transient effects were found. In the first 10 minutes after exposure, only a hyperexcitation at higher micromolar concentrations is observed. The inhibition seen at 100 μM after longer exposure was not visible here. A possible explanation might be that the inhibitory effects on VGCCs need a longer time to manifest, while the hyperexcitation occurs immediately after exposure. Interestingly, for endosulfan this hyperactivity is more pronounced in females than in males. To our knowledge, this is the first time *in vitro* sex-specific acute effects have been found after organochlorine exposure.

In vitro to *in vivo* extrapolation by Croom et al. (2015) showed that rat brain lindane levels associated with seizures or convulsions are similar to concentrations that increased mean firing rate (Croom et al., 2015). Humans and rats are reported to have a similar threshold for lindane-induced seizures. This demonstrates that *in vitro* MEA results can be predictive of *in vivo* responses both in rats and humans. Furthermore, another study (Saavedra et al., 2021) found that human cells are more sensitive to lindane and dieldrin exposure than rat cortical cells. In this light, the effects on neuronal activity we found in rats might be conservative compared to actual effects in humans after organochlorine exposure.

Pyrethroids exert their acute neurotoxicity primarily by interacting with voltage-gated sodium channels (Johnstone et al., 2017; Magby and Richardson, 2017; Shafer et al., 2005), while other targets include voltage-gated calcium channels, potassium channels, and chloride channels (Weiner et al., 2009; Zheng et al., 2019). Pyrethroids can be subdivided into type I and type II pyrethroids, depending on their structure and the clinical symptoms they induce (Baskar and Murthy, 2018; Johnstone et al., 2017; Shafer et al., 2005; Weiner et al., 2009). Type I pyrethroids cause repetitive firing, while type II pyrethroids initially cause prolonged repetitive firing followed by a depolarizing block and cessation of action potential generation (Johnstone et al., 2017; Shafer et al., 2005; Zheng et al., 2019).

Exposure to the type I pyrethroid permethrin and the type II pyrethroid alpha-cypermethrin had opposite effects, with permethrin stimulating neuronal activity and alpha-cypermethrin strongly inhibiting neuronal activity at concentrations exceeding 10 μM. The increase in activity after permethrin exposure was more pronounced in males than females. The inhibition seen after alpha-cypermethrin exposure is in line with earlier studies that have investigated the effect of this compound on neuronal activity (Baskar and Murthy, 2018; Johnstone et al., 2017; Saavedra et al., 2021). Baskar and Murthy (2018) found that permethrin also inhibited neuronal spiking, but less potent than alpha-cypermethrin. Changes found in mean burst rate were comparable to that of mean spike rate. However, in the study by Baskar and Murthy (2018) both alpha-cypermethrin and permethrin were added cumulatively with increasing doses, making it difficult to compare these results to our own. In our study, each well was exposed to only one condition to prevent possible effects of cumulative dosing, like (de)sensitization of receptors and/or ion channels.

Research by (Johnstone et al., 2017; Saavedra et al., 2021; Valdivia et al., 2014) does show an increase in neuronal activity after permethrin exposure, which is in line with our results. Johnstone et al. (2017) postulate that in many earlier studies on the effects of pyrethroids on neuronal activity, GABAA receptors were blocked. This causes a high baseline firing rate in the network, which might be the reason the increase caused by permethrin is not observed in earlier studies. The depolarization-dependent block of action potentials caused by cypermethrin would still be observed if GABA-receptors are blocked (Johnstone et al., 2017).

The use of multiple neuronal activity parameters greatly expands our knowledge of activity patterns following acute insecticide exposure. Most studies on MEAs focus on general parameters of neuronal activity (i.e. number of spikes and (network) bursts). Here, we show the results from ten parameters identified by a PCA to contribute most to the concentration-dependent variation. The parameters discussed overlap with those reported by (Kosnik et al., 2020) and will provide a more robust assessment of neurotoxicity than general activity parameters alone. The difference in (network) bursting patterns seen after exposure to CPF and CPO is a clear example that shows it is important to look at other parameters than those related to general activity.

The current study showed effects found after 30-minute exposure, which is sufficient to mimic acute intoxication. However, most real-life exposure takes place over a longer time. Since most pesticides can also function as EDCs, the sex-specific effects found here may be even more common after long-term chronic exposure. Additionally, studies show that acute exposure effects can influence future behavior *in vivo* (Eriksson et al., 1992; Lee et al., 2015b, 2015a). Therefore, it might be interesting to investigate if the *in vitro* acute effects found here persist over time after chronic exposure.

It is important to note that, while some of the previously observed sex-specific effects in *in vivo* studies can be due to sex-specific differences in metabolism or distribution, the acute sex-specific effects found in our *in vitro* study cannot be related to sex-specific ADME differences. Hence, it seems that there are subtle differences in neuronal characteristics between female and male cortical cultures, such as possible differences in the amount of specific neurotransmitter receptors or ion channels, that remain undetected until a chemical challenge.

To conclude, our study shows that acute exposure to a variety of insecticides has different effects on neuronal activity. Moreover, it

demonstrates that MEA recordings are suitable for detecting sex-specific effects *in vitro*. Using MEA recordings as an *in vitro* assay to screen neurotoxic compounds will greatly reduce the number of *in vivo* studies that are costly and require a large number of animals.

CRediT authorship contribution statement

Anneloes Peerdeman: Investigation, Formal analysis. **Lennart van Melis:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Remco H. S. Westerink:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Aart de Groot:** Software. **Regina van Kleef:** Methodology, Investigation. **Eva Huiberts:** Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest. Given his role as Editor in Chief of NeuroToxicology, Remco H.S. Westerink had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Dr. Pamela J. Lein.

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.neuro.2024.04.004.

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