Endocrine-Disrupting chemicals (EDCs): evaluation of sex-specific effects on developmental neurotoxicity using microelectrodes array (MEA) recordings

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1. Abstract

Endocrine-disrupting chemicals (EDCs) are known to disturb normal hormone signaling. Disruptors of hormone pathways, such as cypermethrin, permethrin, benzyl butyl phthalate (BBzP) and perfluorooctane sulfonate (PFOS) are associated with human health adverse outcomes, mainly during the neurodevelopmental window. However, there is a knowledge gap that links the neurodevelopmental toxicity of these chemicals and their endocrine disruptor activity. A key aspect of this study is that hormone receptors are differentially expressed between females and males. Thus, the current study aimed to assess the sex-specific effects on the development of neuronal activity of endocrine disrupting chemicals. To do so, rat cortical cells were exposed for 21 days in vitro to different concentrations of EDCs, and the neuronal activity was measured using 48-well microelectrode array (MEA) recordings. Our findings suggest a sex-specific effect after the exposure to PFOS showing an increase of network activity over time only in females. Besides, permethrin presents a decrease in neuronal activity in females and an increase in males. Moreover, results represent a concentration-dependent decrease on neuronal activity after exposure to cypermethrin over time. These findings suggest a relationship between these compounds and the effects on neurodevelopmental effects. Therefore, this research provides a further step to establish scientific evidence on the neuronal toxicity caused by the endocrine disruptor effects. However, more studies are needed to improve the lack of information on the impact of EDCs on neuronal activity and human health.

2. Layman summary

In our body, there is a network of glands, and organs called the endocrine system that produces, stores and secretes hormones. Usually, hormones fit into specific receptors and regulate the normal processes of our body such as growth, metabolism, digestion, or sexual function. However, in different products such as plastics, pesticides, or even in the water or food, we can find hundreds of dangerous chemicals called endocrine-disrupting chemicals or EDCs. EDCs get into the body, mimic the action of the hormones, and link to the hormone receptor instead. Consequently, they disturb the normal biological process and negatively affect human health mainly in the neuronal system. The abnormal function of hormones in the brain, especially during neuronal growth, may produce neurodevelopmental outcomes. It has been shown that hormones and EDCs produce different effects in the brain of males and females. However, there is insufficient knowledge of the neuronal effects produced by EDCs. Thus, this study focuses on assessing the effects of potentially hazardous EDCs on the development of the brain in each sex. To investigate so, females and males rat neuronal cells were grown in microelectrode array (MEA) plates and exposed to different concentrations of EDCs for 21 days. The MEA plates contain electrodes that can record the electrical neuronal activity of the cell culture. Results demonstrate that MEA is a valid method to test the changes in neuronal activity after exposure to these chemicals. The current findings found differences between sexes after exposure to PFOS, that presented an increase on the neuronal activity in females. Permethrin showed a decrease on neuronal activity in females and an increase in males. Besides, cypermethrin produced different neuronal effects at different concentrations, reducing the neuronal activity at the highest concentration over time. This study is important to gain insight into the impact of these chemicals on our brain growth. However, more knowledge is needed to ensure a causal link between the hormonal disruption activity and the brain development effect. Therefore, there is a need to test EDCs and improve the methods to evaluate them. Governments and regulatory agencies should develop strategies towards regulations of EDCs to protect human health and provide information to the public on the products that may contain these chemicals and their hazardous properties.

3. Keywords

Endocrine disrupting chemicals (EDCs); microelectrode array (MEA); neurodevelopment; neuronal activity; chronic exposure

4. Introduction

In recent years, there has been a growing global concern over the effects that may result from exposure to chemicals that potentially disrupt the endocrine system in wildlife and humans. Endocrine-disrupting chemicals (EDCs) are defined as agents that may interfere with the synthesis, secretion, transport, binding, or elimination of natural hormones in the body (Andersson et al., 2018). Endocrine disrupting chemicals act by linking with the hormone receptor directly, stimulating (agonists) or inhibiting (antagonists) them, or indirectly, by altering hormone synthesis (Combarnous, 2017).

There are chemicals well-known to act as EDCs. For instance, the well-documented pesticide DDT, used worldwide since the 1940s and banned in Europe in the 1970s after being associated with the decline of the alligator´s reproductive in the Lake Apopka (Padayachee et al., 2023; Woodward et al., 1993). DDT and its metabolite DDE act by mimicking the action of estrogen and androgens and may cause hormone-related cancers including breast and prostate cancers (Cohn et al., 2015; Lenters et al., 2019). In addition, exposure to PFOS (polyfluoroalkyl substance, PFAS) is associated with the disruption of Liver Oxysterols (LXR) receptors (Lupu et al., 2020). PFOS was used as a protectant of commercial and industrial products but is banned in the EU since it is persistent in the environment and drinking water (Pierozan & Karlsson, 2021). Although there are examples of chemicals that have evidence of hormone-like activity, there is still a lack of knowledge of a myriad of potentially dangerous chemicals used worldwide and implicated in the disruption of the hormone system. Another potentially dangerous EDC widely used in everyday products is BBzP. BBzP is found in plasticizers, additives, and solvents, and it is associated with disruption of the prostaglandin E2 (PGE2) receptor (Land et al., 2021). The pyrethroid family is also implicated in endocrine disruption. Pyrethroids are synthetic insecticides utilized in agricultural households and medical formulations and act by prolonging the time voltage-sensitive Na^+ channels (VGSCs) are opened, thereby increasing the Na^+ influx inducing hyperexcitation. This family is broadly divided into type I pyrethroids, which lacks an α-cyano group and type II pyrethroids, which contain an α-cyano group. Type II pyrethroids open Na^+ channels for a longer time period compared to type I pyrethroids (Johnstone et al., 2017a), which can cause depolarization of the cell membrane and loss of neuronal activity. Secondary consequences of pyrethroid toxicity are related to gamma-aminobutyric acid (GABA) target sites and thyroid hormone disruption (Kunno et al., 2021; Leemans et al., 2019) . Exposure to EDCs is associated with several diseases related to prenatal growth, glucose metabolism, obesity, puberty, fertility, and carcinogenesis (Street et al., 2018). Even though the brain is dependent on hormones and highly susceptible to chemicals, especially during development, one of the less studied outcomes is how EDCs are linked with developmental neurotoxicity (DNT). Evidence for associations between EDCs and DNT has been shown. For instance, results have suggested that bisphenol-A and phthalates impact neurodevelopmental outcomes (Welch et al., 2022; Xu et al., 2020). Early life exposure to BBZP has a negative impact on cognition and neurobehavior (Zhang et al., 2019). Hence, there is an urgent need for risk assessment to identify harmful EDCs better and thereby implement legal frameworks that attempt to expose chemical regulations actions.

To understand the relationship between EDCs and DNT it is essential to look into hormone signaling and their role during critical windows of brain differentiation. A key driver in this study is that hormone receptors are expressed in a sex-dependent manner during neurodevelopment (Sundukov, 2006). Androgens and estrogens, also called sex steroids, have a crucial role in brain development and sexual differentiation. Evidence shows that sex steroids are involved in the neurogenesis process and are implicated in neurodevelopmental pathogenesis, such as schizophrenia and depression (Heberden, 2017; Hill et al., 2012; McEwen & Milner, 2017). Moreover, other hormone-signaling pathways play important roles in neurodevelopment. Thyroid hormone (TH) is essential for brain development and maturation (Mughal et al., 2018), and alterations in TH signaling may cause neurodevelopmental impairments (Gilbert et al., 2012; Moog et al., 2017). Expression of receptors such as LXR has been demonstrated to be involved in the modulation of brain function, regulation of glial cell functions and disruption of this pathway can increase the risk of developing neurodegenerative disorders (Mouzat et al., 2019; Song et al., 2022). Moreover, PGE2 plays an essential role in cell proliferation and neuronal differentiation of stem cells (Mouzat et al., 2019).

To evaluate the developmental neurotoxicity of PFOS, BBzP, cypermethrin, and permethrin, we have measured neuronal network activity after exposure to these compounds using multi-well microelectrode arrays (MEAs). MEA is a non-invasive measurement that allows the recording of electrophysiological activity of cultured excitable cells in vitro, such as neurons (Hondebrink et al., 2016; Shafer, 2019). Rat primary cortical cultures are suitable for detecting changes in neuronal activity after chronic exposure to chemicals since this culture compromises diverse cell types such as astrocytes and glutamatergic neurons and molecular targets like GABA receptors and voltage-gated calcium channels (VGCCs). Although using human cells model such as iPSC-derived neurons may avoid the interspecies estimation error, in this study rat neurons model is used as it is a less-expensive and short culture duration. Besides, minor differences between human iPSC-derived neurons compared to rat primary cortical cultures were demonstrated (Tukker, Wijnolts, et al., 2020; Gerber et al., 2021). As exposure to EDCs may occur throughout life and has particularly important effects during early life, a long-term exposure assay with developing rat cortical cells is a suitable neurotoxic screening method to study the relationship between ED and DNT. In this study, cells are cultured for 28 days, the maximum time these cultures can be maintained without loss of neuronal activity. The spontaneous activity is detected as the number of spikes, bursts, and network bursts. The difference between these three parameters is that the number of spikes characterizes the action potential of the neuronal cells. The number of bursts represent the spikes produced in a single electrode over a short time, and the network bursts are the bursts recorded in different electrodes of the same well (Gerber et al., 2021a). MEA recordings provide an efficient screening method to predict the developmental neurotoxicity effects of chemicals. Moreover, MEA is an in vitro toxicity test that allows to assess low-cost, non-invasive, reduces animal-use, and higher throughput studies (Kosnik et al., 2020; McConnell et al., 2012). Since it is unclear how endocrine disrupting chemicals can potentially induce DNT, there is a need for risk assessment evaluation to identify the effects EDCs have during neurodevelopment. Therefore, the present study aims to establish a causal link between ED and neurodevelopmental toxicity related effects. To this end, the

neuronal network activity of human-relevant compounds (phthalates, pyrethroids, and perfluoroalkyls) with endocrine disrupting properties was studied using MEA recordings.

5. Materials and methods

Chemicals and samples

Four compounds were used in the study: perfluorooctanesulfonic acid (PFOS), benzyl butyl phthalate (BBzP), cypermethrin and permethrin. PFOS (>98% purity) and permethrin (98,3% purity) were obtained from Fluka (Zwijndrecht, The Netherlands). BBzP (98% purity) and α-Cypermethrin (≥98.0% purity) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Further information about the compounds used is summarized in **table 1**.

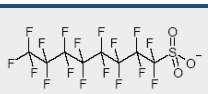
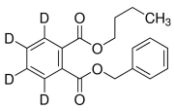
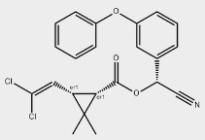
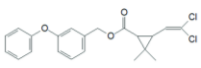
Group	Compound	Chemical formula	Molecule	ED	MW	Lot	Company	CAS RN
Perfluoro chemicals (PFCs)	Perfluorooctanesulfonic acid (PFOS)	$C_8HF_{17}O_3S$		LXR, AR, TR	538.22 g/mol	1246519	FLUKA	1763-23-1
Phthalates	Benzyl butyl phtalate (BBzP)	$C_{19}H_20O_4$		PGE2R	312.36 g/mol	MKCM1987	SIGMA-ALDRICH	85-68-7
Pyrethroids	Cypermethrin (Cyp)	$C_{22}H_{19}Cl_2NO_2$		TR	416.30 g/mol	BCBS3967V	SIGMA-ALDRICH	52315-07-8
Pyrethroids	Permethrin (Perm)	$C_{21}H_{20}Cl_2O_3$		TR, VDR, PPAR	391.29 g/mol	SZBB206XV	Fluka	52645-53-1

Table 1: Summary table of the samples tested. Overview of the chemical group, the formula and molecule, potential receptor involved in ED activity, molecular weight, number of lot of the compounds used, company manufacturer, and CAS number. *ED= endocrine-disrupting receptor; LXR= Liver X receptor; AR= androgen receptor; TR= thyroid hormone receptor; PGE2R= prostaglandin E2 receptor; VDR= Vitamin D3 receptor; MW= Molecular weight; CAS RN=chemical abstracts service registry Number.

The stock solutions were prepared by adding Dimethyl Sulfoxide (DMSO) and were stored in sterilized amber glass vials at 4°C. DMSO stock solutions were diluted 1:1000 in medium to get the final exposure concentrations, 0.01 µM to 100 µM through serial dilutions.

Microelectrode arrays (MEA)

Microelectrode arrays (MEA) consist of a non-invasive platform to record, amplify and analyze the electrophysiological activity of excitable cells in vitro. 48 multi-well BioCircuit MEA plates with an opaque well bottom were used in the functional neurotoxicity experiments. Each well integrates an electrode array of 16 gold microelectrodes (40–50 µm diameter; 350 µm center-to-center spacing) (Tukker et al., 2018). MEA plates were placed into the system Maestro Pro multi-well microelectrode array (Axion Biosystems, Axion Integrated Studio (AxIS) software version 2.5.2) that integrated CO_2, heating, and temperature system control used to record the electrical activity of neurons.

Cell culture

All experiments were performed according to Gerber *et al.* (2021). Performing experiments requires careful planning, pre-treatment, and washing of the 48-well MEA plates. MEA plates are re-used up to four times, and washing steps under sterile conditions are necessary. 48-wells MEA plates require to be coated at least one day before the isolation with 0.1% polyethyleneimine (PEI) to allow cells to be attached to the wells. Rat primary cortical cells were cultured on MEA plates. The cultures include diverse cellular in vivo cells, such as excitatory and inhibitory neurons and astrocytes used to screen neuronal activity. The isolation procedure was performed by professionals with knowledge and insight into animals used for scientific purposes. Males and females Wistar Rat pups on postnatal day 0- 1 (Envigo, Horst, the Netherlands) were decapitated with scissors. Cortices from brains were placed in a petri dish containing ice-cold dissection medium (500 mL Neurobasal®-A(NBA) medium (phenol red-free), 14g sucrose (28/100mL), 1.25 mL L-glutamine (200mM), penicillin-streptomycin (5000 U/mL), 50 mL B27 Plus Serum-Free Supplement (50X), 5 mL glutamate solution (3.5mM) and pH adjusted to 7.4). Once cortices were triturated and centrifuged for 5 min at 800 rpm, suspension of 2×10^6 cells/mL was adjusted. The cells were seeded by 50 µl drops that were added in the center of each well of 48-well MEA plated already coated with PEI. Cells were kept for 2 hours in a humidified incubator at 37ºC with 5% CO_2 to adhere to the array surface. After adding dissection medium (450 µl/ per well), on DIV 4, the 90% of the medium was replaced with culture medium (dissection medium without l-glutamate). Cells were kept in a humidified incubator until the start of the experiment on DIV 7.

Animal experiments were approved by the Ethical Committee for Animal Experiment of the University of Utrecht and performed in agreement with Dutch law and the European Community directives regulating animal research (2010/63/EU).

Chronic exposure experiment

Developmental exposure experiments started on DIV 7, measuring 30-min baseline MEA activity. Before the measurement, Axion software settings were set up. 768 simultaneous live recordings were sampled with a frequency of 12.5 kHz, a gain of 1200×, and a band-pass filter of 0.2-5 kHz creating raw (.RAW) data files. After ~20 minutes of baseline recording, the number of active electrodes per well was counted at the Spike Plots module and noted in an exposure schedule. The

number of bursting electrodes per well should be ≥4 to consider the detection of neuronal activity. At least six wells were used as control exposure.

During the process, 48-well MEA plates were placed in an incubator at 37°C with 5% CO_2. At DIV 10, 14, 17, 21, and 24, 30-min MEA measurements were recorded (after equilibrating MEA plates in Maestro for ~5 minutes). To save time, exposure solutions of the test compounds were prepared during MEA recordings. Exposure solutions were warm and kept at 37°C. Since there are changes in the water volume produced because of the evaporation, the volume of each well was corrected at each half-medium change. To correct for the volume, we added 20 µL of sterile water to the outer wells and 5 µL to the inner wells. Once the water volume was adjusted, we replaced 50% of the medium change. First, 250 µL of the medium was removed from all the wells. Second, 250 µL of the exposure dilution was added carefully. Since in the first exposure (DIV 7; baseline), the cells were not exposed to the compound yet, the concentration of the exposure solution added in the medium change doubled the amount of the desired exposure concentration used in the next medium changes. At DIV 28, we started another 30 min recording. After the recording, cell viability was analyzed.

Cell viability assay

On DIV 28, the viability of the cell culture was assessed using the Alamar Blue (AB) assay. AB provides a rapid method for assessing mitochondrial metabolic activity of the primary cortical after exposure to the compounds. The purpose of AB bioassay in the chronic exposure experiment was to determine whether changes in neuronal activity were caused by the cytotoxic effects of the compounds used. AB is based on the ability of viable cells to reduce resazurin (non-fluorescent blue reagent) to a resorufin (fluorescent pink compound). Resazurin permeates into the cells and in the mitochondria, it is converted to resorufin by accepting electrons of NADPH, GADH, FMNH, NADH, and cytochromes. The Redox reaction causes a shift in color from blue(oxidized) to pink (reduced), being easily measured using colorimetry or fluorometry reading at 590 nm. Once the last MEA recording was measured, the culture medium was replaced by 300 µL of Alamar Blue (25uM). To obtain the concentration of 25uM working solution, AB 5mM was diluted 1:200 in 37°C prewarm HBSS (1x). To prepare HBSS 1x, HBSS x10 was diluted in distilled water. MEA plates and the AB remaining working solution were incubated for 1.5 hours at 37°C with 5% CO_2 and 95% air atmosphere. Afterward, 200 µl of the AB from each well were transferred from the 48-well plate to a transparent 96-well culture plate. Each row of the 96-well plate corresponded to each concentration. At least 3 wells of one row were used as a blank control adding 200 µl/per well of AB incubated. After that, the fluorescence was measured with a microplate reader (Tecan Infinite 200 reader, Männedorf, Switzerland). Excitation was set up to 540 nm and emission to 590 nm and the area filled was selected. Before introducing the plate in the Tecan, bubbles in wells were carefully checked and eliminated.

Data analysis

MEA recordings were saved in .RAW data files in the Maestro Pro multi-well microelectrode array. After MEA recordings were completed, Integrated Studio (AxIS) software was used to generate AxIS spikes files from the .RAW files. Spikes were detected using the AxIS spike detector (Adaptative threshold crossing) with an optimal threshold of 7*standard deviation (SD) to differentiate between spikes and the background noise. Pre and post-duration of the spikes were 3.6/2.4 ms for each electrode. To return the signal below the threshold before detecting an additional spike, the "Detect only crossings" option was selected. Coincident artifacts were removed from the analysis, and the "spike counting interval" was set to 1 s.

The software NeuralMetrics Tools (v 3.1.7, Axion BioSystems) was used to analyze .spk files generated, identifying bursts and network bursts. The file segment analyzed was the last 1200 seconds of the recording since it is the most stable timeframe, and only active electrodes (6 spikes/min) were included. Spikes that were detected on multiple electrodes at the exact time were seen as coincident artifacts and were excluded from the analysis. To identify bursts, spikes were compared to the expected collection of spikes under a "Poisson surprise" distribution in a given period of time, using a minimun surprise of 10. The network burst was analyzed using an adaptive threshold method with a minimum of spikes of 40 and a maximum electrode per well of 15%. The NeuralMetrics Tools generated .csv files with the raw values for each parameter for each recording. The .csv files obtained were combined using a custom-made Excel macro. Raw values for baseline and exposure recordings were grouped per condition in an excel template. Only wells with more than four bursting electrodes were included. Experimental values that exceeded mean ± 2×SD were considered outliners and were removed. To calculate the treatment ratios per well, raw values were normalized per point (DIV 7, 10, 14, 17, 21,24, and 28) to time-matched average control value (set to 100%). Finally, the data represented average values derived from at least 2 independent experiments (N). Results were presented as mean % ± standard error of the mean (SEM).

In the cytotoxicity assay, fluorescence values were normalized ((Fluorescence value exposed well∗ 100/Mean fluorescence value control) = mitochondrial activity (% of control)) setting the mean control value to a 100% metabolic activity. The values of the exposure were compared with the average of the control. Data from different plates were combined excluding values in which treatment ratio was more than the mean ± 2×SD (for each condition).

Statistical analysis

All data were analyzed using GraphPad Prism 9 (GraphPad Software, San Diego, California, USA). A two-factor ANOVA test was used to calculate changes in the neuronal activity and cytotoxic effects for each compound. Tukey´s test was performed to assess a multiple significant comparison. In the cytotoxicity assay, cell viability was compared in males and females. In the chronic assay, MNS, MNB, and MNNB results were compared independently in time-dependent and concentration-dependent manner, in which control group was the DMSO exposed one.

Furthermore, sex-specific effects were assessed by mixing the effects of concentration and time and comparing them between males and females. Once we analyzed the data, graphs were obtained. Benchmark response (BMR) was based on the average variation in control experiments at DIV 7. The resulting data were presented as mean ± standard error of the mean (SEM) normalized to the control. A p-value< 0.05 was considered statistically significant.

6. Results

In this study, following the procedure outlined above, rat cortical female and male cells were exposed to one of four compounds (PFOS, cypermethrin, permethrin, and BBzP) at 0.01-100 µM, for 21 days (day in vitro (DIV) 7-28). Neuronal activity was recorded at several time points and alterations in spontaneous neuronal activity were expressed as treatment ratios (% change compared to time-matched control) by assessing the number of spikes (MNS), bursts (MNB), and network bursts (MNNB). Cytotoxicity was assessed using Alamar Blue (AB) assay.

Changes in the neuronal activity pattern after chronic exposure to PFOS

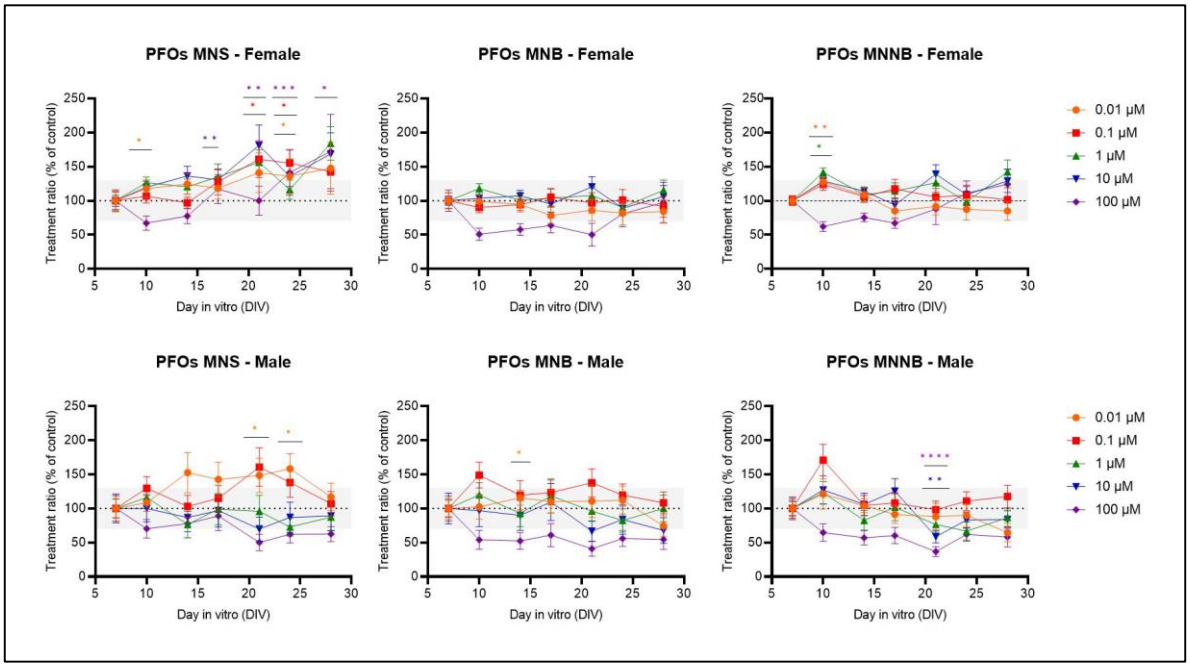


Figure 1. Developmental effects of PFOS on rat cortical neuronal activity. Concentration-response relationship for PFOS (0.01-100 µM) is tested over time during chronic (DIV7-28) MEA experiments. Neuronal activity is represented as the number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for female (top graphs) and male (bottom graphs) cells. The results are presented as mean treatment ratio normalized to control (DMSO set to 100%) ± SEM from the number of wells (n) between 14 and 16 in females and 11 and 15 in males. Two independent experiments(N) were performed and normalized to DIV 7. The grey area shows a 30% benchmark response derived from the average variation in control experiments at baseline (DIV 7). Asterisks represent data points with a significant difference compared to the control ($p \leq 0.05$). The color of the asterisks indicates which concentration is significantly affected (orange for 0.01 µM; red for 0.1 µM; green for 1 µM; blue for 10 µM; purple for 100 µM). The lack of asterisks means that no significant data was founded.

As can be seen in **Figure 1**, minor differences in MNB and MNNB were observed between male and female pattern activity after the exposure at the DIV 7. Nevertheless, MNS presents a noticeable difference between sex showing a tendency in females to increase in a time-dependent manner the activity. Whereas parameters of male neuronal activity remain mostly constant during the time.

In females, spontaneous neuronal activity on MNS suggests that all concentrations present a gradual increase in the activity from DIV 10 onwards, raising 150% of the activity on DIV28. In **table 6** (see appendix), significant effects can be founded. Contrary to these results, bursts and network activity present only minor differences, remaining constant with no remarkable changes over time.

Male results demonstrate that exposure to PFOS at all concentrations over time has a negligible effect on spontaneous electrical network activity (measured by MNS, MNB, and MNNB, no significant effect). While at the highest concentrations (10 and 100 µM), PFOS evokes a reduction of the activity, at the lowest concentration (0.01 µM) raises the activity.

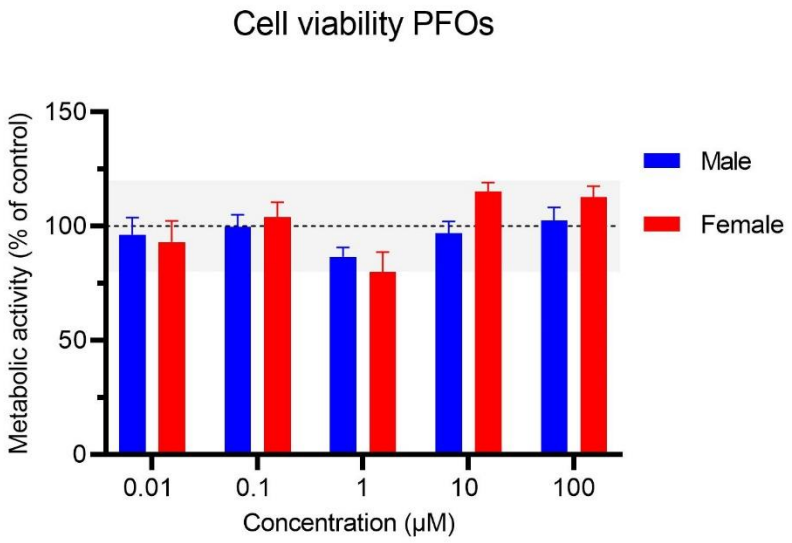


Figure 2. Cell viability after developmental exposure to PFOS. Cytotoxic effects of PFOS (0.01-100 µM) were determined using Alamar Blue (AB) on DIV28 after the chronic exposure experiment. Metabolic activity is evaluated per concentration normalized to DMSO controls (DMSO set to 100%) ± SEM from n=14-16 in females and n=11-15 in males, N=2. Mitochondrial activity results were presented in color blue for males and red for females. The dotted line represents 100% of cell viability, and the grey area indicates a 20% benchmark response derived from the average variation in control experiments. Results were considered statistically significant when p-value<0,05.

Mitochondrial activity was assessed using Alamar Blue (AB) assay based on a redox reaction. As can be seen in **Figure 2**, PFOS shows no significant cytotoxicity effects in females or in males for the concentrations studied.

Neuronal activity after chronic exposure differs from type I and type I pyrethroids

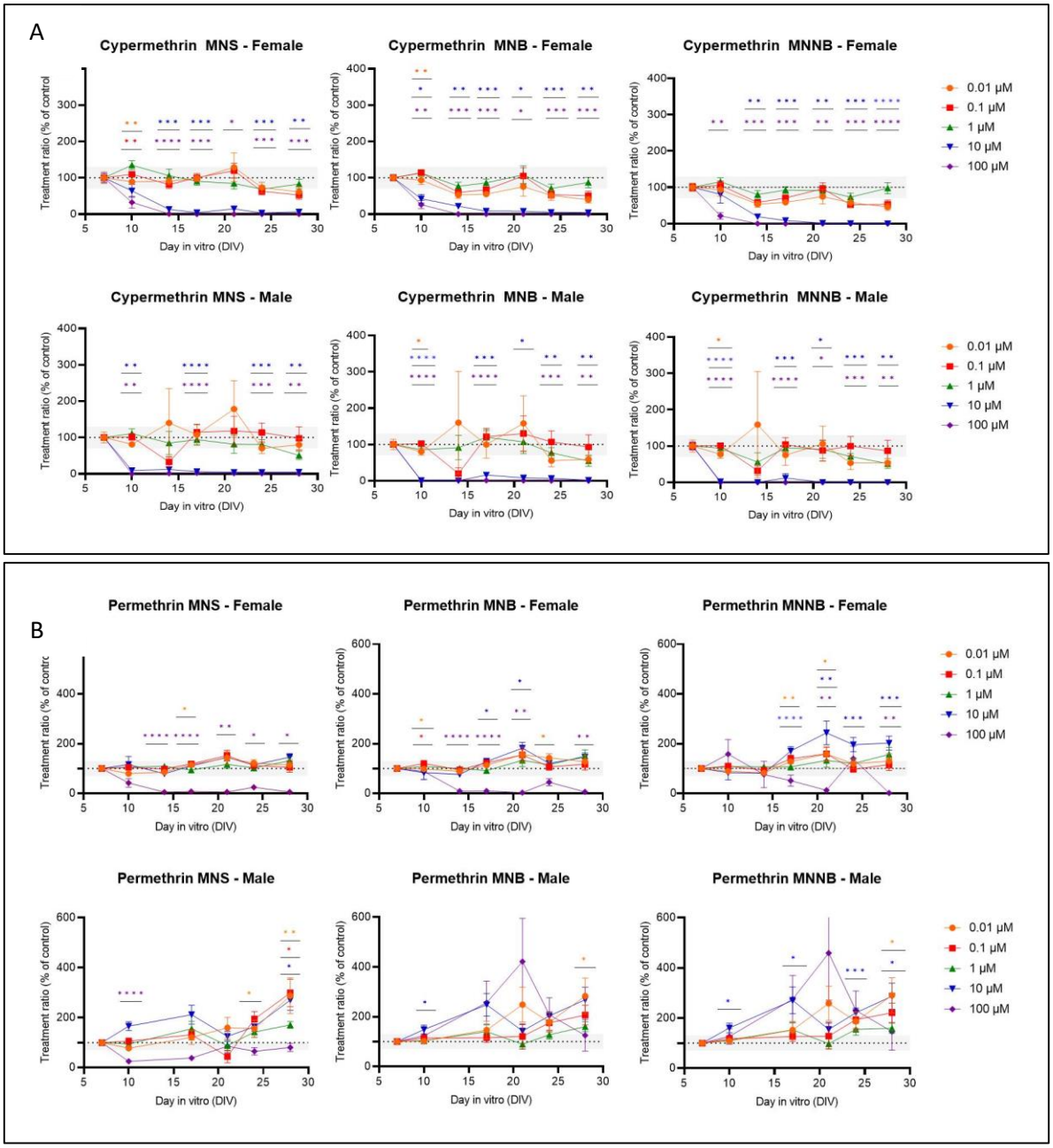


Figure 3. Developmental effects of cypermethrin (A) and permethrin (B) on rat cortical neuronal activity. Concentration-response relationship for cypermethrin and permethrin (0.01-100 µM) is tested over time during chronic (DIV7-28) MEA experiments. Neuronal activity is represented as the number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for female (top graphs) and male (bottom graphs) cells. The results are illustrated as mean treatment ratio normalized to control (DMSO set to 100%) ± SEM. The number of wells assessed in cypermethrin was n=15-16 (females) and n=14-16 (males), while in permethrin n=14-16 in females and males. Two independent experiments were performed and normalized to DIV 7. The grey area shows a 30% benchmark response derived from the average variation in control experiments at baseline (DIV 7). Asterisks represent data points with a significant difference compared to the control ($p \leq 0.05$). The color of the asterisks indicates which concentration is significantly affected (orange for 0.01 µM; red for 0.1 µM; green for 1 µM; blue for 10 µM; purple for 100 µM). The lack of asterisks means that no significant data was founded.

Cypermethrin and permethrin are pyrethroids with different chemical formulas. Whereas permethrin (type I pyrethroid) lacks an α-cyano moiety, cypermethrin (type II pyrethroid) contains an α-cyano moiety at the alpha-position (**Figure 4**). Consequently, it raises the question of whether the effect after chronic exposure to both compounds is equivalent or different. Rat cortical cells were exposed to different concentrations (0.01-100 µM) of permethrin and cypermethrin. We Spontaneous neuronal activity was measured for 28 days, and results were compared.

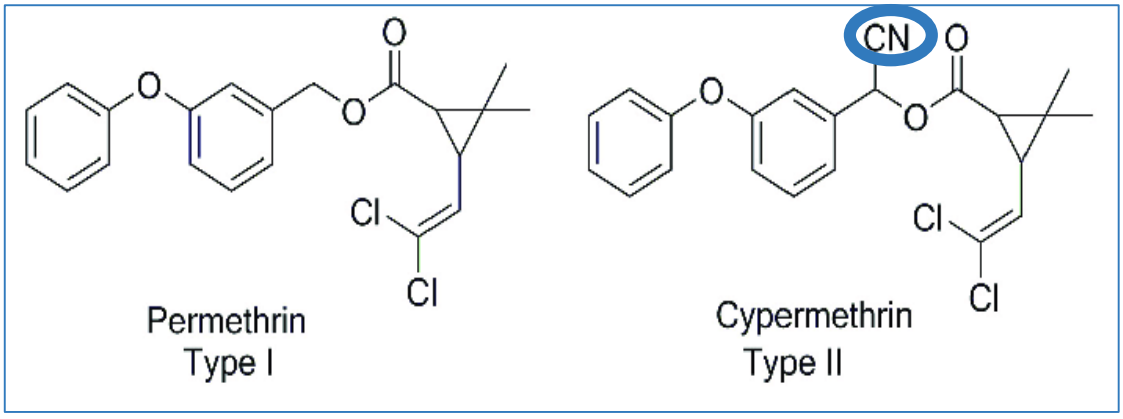


Figure 4. Chemical structures of permethrin (Type I pyrethroid) and α-cypermethrin (Type II pyrethroid).

It is visible comparing **Figure 3.A** and **Figure 3.B**, that neuronal activity patterns differ from both compounds. The results demonstrate that permethrin has a concentration-dependent decrease in spontaneous neuronal activity over time in both sex. Whereas permethrin effect reveals a slight time increase in neuronal activity only in males.

Figure 3.A presents the treatment ratios after cypermethrin exposure showing almost the same activity curve in males and females with no major differences between parameters. In males, the activity drops to almost full inhibition at 10 µM and complete inhibition at 100 µM on DIV10 onwards (statistical significance $p<0.05$). Similar to these results, the highest concentration in females decreases gradually on DIV10 inducing full inhibition at 100 µM on DIV14 and at 10 µM on DIV 17. These results are significant (see supplementary data table 4). Small noticeable effects were presented in females and in males after 0.01, 0.1, and 1 µM exposure to cypermethrin on the number of spikes, bursts, and network bursts. The activity remains stable at the time with a slight decrease on DIV 28 in both sexes. Exceptionally, in graph **3.A** a modest peak can be observed after the exposure to cypermethrin in male cells at 0.01 µM on DIV 21 (MNS and MNB) and DIV 14 (MNB and MNNB). However, these random effects might be considered not relevant to our results. Moreover, after 0.1 µM exposure, males presented a drop in all parameters on DIV 14. However, these results were not significant (**table 4** appendix).

Alterations in neuronal activity are found in **figure 3.B**, after chronic exposure to permethrin at 0.01-100 µM concentration. Males and females present different tendencies in the activity on all three parameters. Male shows a sharp time-dependent increase in the spontaneous spikes rate, with a peak in the DIV28 (almost 300% of the activity at 0.01, 0.1, and 10 µM). At the highest concentration,

the number of spikes declined nearly full inhibition at DIV 10, and at DIV 14 the activity was recovered. Female rat cells exposure to 100 µM completely inhibits MNS and MNB on DIV14 (significant p<0,05). In parallel, the rest of concentrations remain constant on time, raising only a bit on DIV 28. Further significant results can be observed in **table 5** in the appendix.

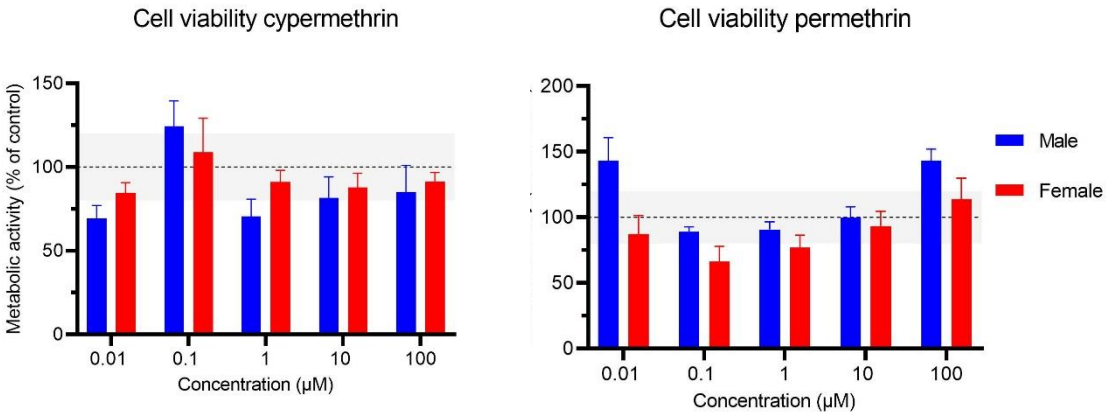


Figure 5. Cell viability after developmental exposure to cypermethrin (left graph) and permethrin (right graph). Cytotoxic effects of cypermethrin and permethrin (0.01-100 µM) were performed using Alamar Blue (AB) on DIV28 after the chronic exposure experiment. Metabolic activity is evaluated per concentration normalized to DMSO controls (DMSO set to 100%) ± SEM from n =15-16 (females) and n = 14-16 (males) for cypermethrin and n =14-16 in females and males for permethrin, N=2. Mitochondrial activity results were presented in color blue for males and red for females. The dotted line represents 100% of cell viability, and the grey area indicates a 20% benchmark response derived from the average variation in control experiments. Results were considered statistically significant when p-value<0,05.

Figure 5 presents the viability of cells after the cypermethrin (graph on the left) and permethrin (graph on the right) exposure. Mitochondrial activity after cypermethrin exposure was slightly lower in males for all concentrations except 0.1 µM which was higher than in females. However, these effects were not statistically significant. As can be seen in the right graph of **figure 5**, metabolic activity in females resulted in non-significant change for all concentrations. Close inspection of right graph indicates, by contrast, that permethrin presents generally upper metabolic activity in males raising almost 150% of the activity at 0.01 µM and 100 µM. Comparing well with males, females present lower activity at 0.1 µM. Results analyzed indicate no cytotoxicity after the developmental exposure to cypermethrin or permethrin, suggesting that both compounds lack cytotoxic effects in cortical cells at the concentration given.

Chronic exposure to different concentrations of the phthalate BBzP releases differences in neuronal activity

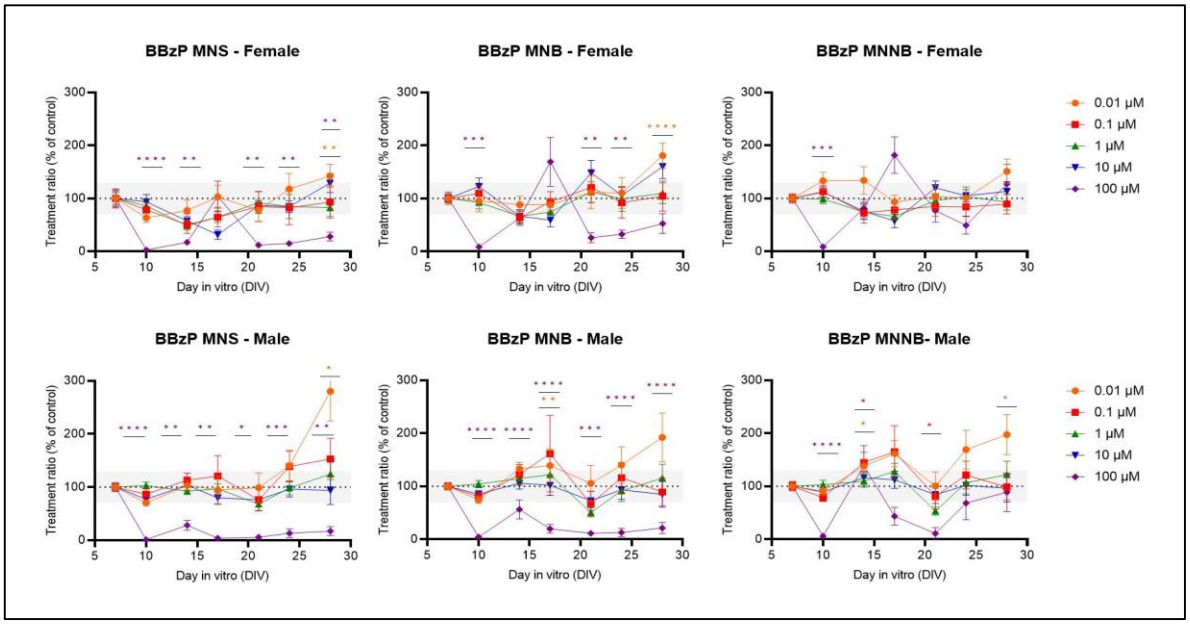


Figure 6. Developmental effects of BBzP on rat cortical neuronal activity. Concentration-response relationship for BBzP (0.01-100 µM) is tested over time during chronic (DIV7-28) MEA experiments. Neuronal activity is represented as number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for female (top graphs) and male (bottom graphs) cells. The results are illustrated as mean treatment ratio normalized to control (DMSO set to 100%) ± SEM from n =15-16 (females) and n = 15-16 (males), N=2. Two independent experiments were performed and normalized to DIV 7. The grey area shows a 30% benchmark response derived from the average variation in control experiments at baseline (DIV 7).
Asterisks represent datapoints with a significant difference compared to the control ($p \leq 0.05$). The color of the asterisks indicates which concentration is significantly affected (orange for 0.01 µM; red for 0.1 µM; green for 1 µM; blue for 10 µM; purple for 100 µM). Lack of asterisks means that no significantly data was founded.

Upon exposure to BBzP, fluctuations in neuronal activity are observed in female and male rat cortical cells over time (See **Figure 6**) .The highest concentration (100 µM) results in a sharp decrease of the activity in both sexes at DIV 10 on the three parameters (statistically significant). After DIV 10, neuronal activity shows different responses in female and male cells. Spontaneous activity in males after exposure at the highest concentration remains almost inhibited over time with a peak of activity (almost 50% of the activity) at DIV 14 on the number of spikes and bursts. The number of networks burst fluctuates during the time points, showing a peak in the activity (response of 100%) at DIV 14 and DIV 28, and full inhibition at DIV 21, although this effect was not significant. Whereas, at 100 µM all parameters in females gradually increase the activity at the DIV 17, showing a noticeable peak on MNB and MNNB that reaches almost 150% of the activity (data not significant). Moreover, the activity is significantly reduced afterward for all parameters at 100 µM (see supplementary data **table 3**). All exposures down to 100 µM except 0.01 µM, presented fluctuation in the activity curve with a slight increase at DIV28 in both sexes with no significant effect. Finally, the lowest concentration (0.01 µM) evokes significant induction of spikes, bursts, and network bursts.

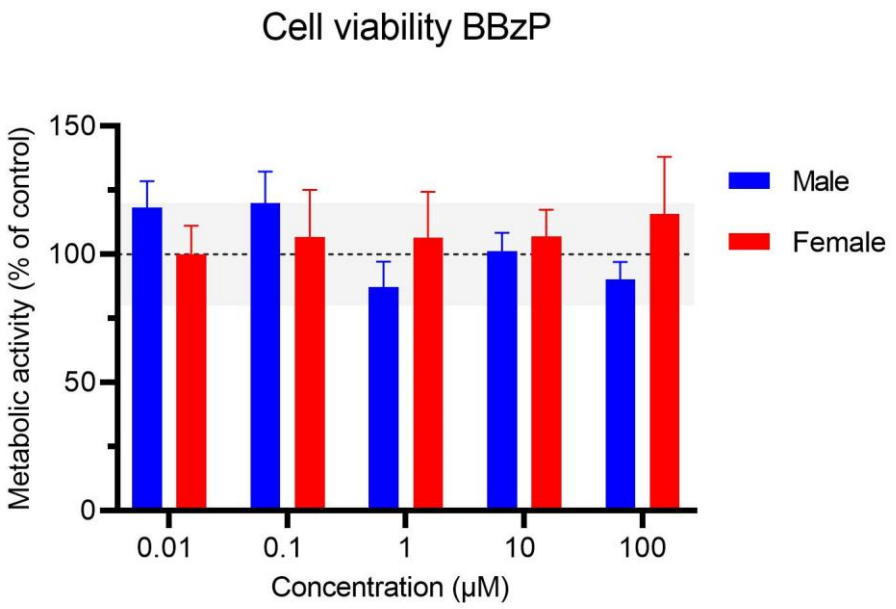


Figure 7. Cell viability after developmental exposure to BBzP. Cytotoxic effects of BBzP (0.01-100 µM) were performed using Alamar Blue(AB) on DIV28 after the chronic exposure experiment. Metabolic activity is evaluated per concentration normalized to DMSO controls (DMSO set to 100%) ± SEM from n=15-16 in females and n=15-16 in males, N=2. Mitochondrial activity results were presented in color blue for males and red for females. The dotted line represents 100% of cell viability, and the grey area indicates a 20% benchmark response derived from the average variation in control experiments. Results were considered statistically significant when p-value<0,05.

As can be seen in **figure 7,** mitochondrial activity assessed by the AB assay, remained stable in all concentrations compared to the control. These findings suggest lacks cytotoxic relevance after the exposure to BBzP at 0.01 µM -100 µM concentrations.

7. Discussion

The present study aims at contributing to the understanding the causal links between EDC exposure and DNT. Accordingly, the effects of four chemicals with endocrine disruptive properties (BBzP, PFOS, cypermethrin, and permethrin) on neurodevelopment were assessed. Micro-electrode array (MEA) recordings were used to evaluate the effects on spontaneous electrical neuronal activity in rat cortical cultures over 21 days of exposure. The overall direction of our results after compound exposure presented changes in the neuronal activity, suggesting that the EDCs used in this study may have neurotoxic effects. In particular, BBzP demonstrated a concentration-dependent increase over time only in females. In the case of cypermethrin, a decrease in the activity was observed at highest concentrations in both sexes. Exposure to permethrin revealed an increase of the activity in males over time but inhibition of the activity at the highest concentration in females. Notably, BBzP exposure resulted in fluctuations in the neuronal activity in both sexes during the time frame. Interestingly, cell viability data indicated no differences in the mitochondrial activity of the cells after

the end of the chronic assay compared to the control at any of the concentrations tested (0.01-100 µM). Results support the assumption that the compounds entailed no cytotoxicity effects.

Remarkably, all of the compounds assessed in this study are allowed to be used in the European Union except for PFOS, which was listed in May 2009 by the Stockholm Convention as a Persistent Organic compound and has been banned since 2020. However, there are no agreements between countries regarding the regulations and prohibitions of these compounds (European Parliament, 2003; Xie et al., 2013). Moreover, although permethrin and cypermethrin have demonstrated highly toxic effects, they were approved to be used as insecticides, acaricides, and pest control products by the European Chemicals Agency (ECHA). Therefore, the study of their potential impacts on the environment and human health is necessary.

In this context, it is essential to underline that PFOS, cypermethrin, permethrin, and BBzP can cross the blood-placenta and brain barrier (Cui et al., 2009; Qian et al., 2020; Trunnelle et al., 2013). Consequently, a differentiated understanding of the neuronal effect of these compounds in early life is necessary. For instance, PFOS may strongly bind to phospholipids, and is accumulated in the brain (Cao & Ng, 2021). A human model study validated that brain contained PFOS in a concentration of 0.01 µM (Fàbrega et al., 2014). Moreover, studies in Greece and Ukraine have shown that the highest concentration of PFOS in the blood of male and female populations of reproductive age was 0.05 µM (Ludwicki et al., 2015; Tukker, Bouwman, et al., 2020). In the case of pyrethroids, research on pregnant Chinese women found a concentration of 0.24 µM in cypermethrin and 0.362 µM in permethrin (Simaremare et al., 2020). Lastly, 0.01 µM BBzP was detected in pregnant women in southern Sweden (Högberg et al., 2008). Studies demonstrated that the blood-brain partition coefficient for cypermethrin, permethrin, and BBzP were 7.94, 1.6, and 8.3, respectively (Quindroit et al., 2019; Sarigiannis, 2019). Although the exact concentration of these compounds in the brain is unknown, these findings suggest that these EDCs may pass through the blood-placenta barrier and be highly distributed to the brain and fetus. The concentration tested in our study varied from 0.01 µM to 100 µM. Thus, the daily exposure concentration of all compounds was within the concentration range tested. It is fundamental to highlight that the highest concentrations (10 µM - 100 µM) exceed the realistic human exposure found in blood and, thus, are not toxicologically relevant. However, it is crucial to test hazards at high concentrations to ensure human exposure safe levels.

Intriguingly, we demonstrated an increase in number of spikes in females after PFOS exposure. Data obtained in previous studies with Zebrafish embryos (Gaballah et al., 2020; Spulber et al., 2014) have demonstrated that developmental exposure to PFOS resulted in neuronal hyperexcitability. Moreover, previous results after acute exposure to PFOS (**figure 7**. appendix), represented an increase in neuronal activity at 100 µM. PFOS is considered a disruptor of the endocrine system since it may act as an LX receptor agonist. Studies with an agonist of LXR (GW3965) suggested that the activation of these receptors induced proper neuronal function (Báez-Becerra et al., 2018). In contrast to this, preliminary experiments after developmental exposure to GW-3965 using rat cortical cells (see **figure 11** in appendix) showed no significant effects on neuronal activity.

Alternatively, other studies recently demonstrated that PFOS could target other non-hormones neuroreceptors. Tukker and colleagues (2020) used MEA recordings in human iPSC-derived neuronal models to demonstrate that PFAs is a $GABA_A$ antagonist. This fact is not surprising, as it has been previously suggested in other studies (Bouwman, et al., 2020; Tukker, Wijnolts, et al., 2020). Further, experiments with Wistar rat pups found activation of NMDA receptors and excess calcium influx after PFOS exposure (Berntsen et al., 2018). Perhaps the lack of findings in our current study suggests that PFOS may target more than one receptor, and it could produce a compensatory mechanism that masks any neuronal effects. Consequently, the relationship between the neuronal effects of PFOS and LX receptors in the brain is unclear.

Of particular interest is that permethrin and cypermethrin, which are type I and type II pyrethroids, have different effects on neuronal activity in our current study. Consistent with Shafer et al. (2005), we observed that the activity of cortical cells is affected differently after exposure to type I and type II pyrethroids. While the cypermethrin exposure suggested a remarkable reduction of the activity, permethrin reveals a slight increase in the activity just in males. Acute exposure results also suggested differences between pyrethroids neuronal activity tendencies. Whereas there was an increase of the activity after 10 µM permethrin in females and males (results can be found in **figure 8** in the appendix), cypermethrin exposure showed a decrease of activity at 10 µM, followed by full inhibition at 100 µM in males and females (Appendix **figure 9**). Intriguingly, our data shows that permethrin demonstrated sex-specific differences in neuronal activity. We observed that cypermethrin presented full inhibition in males and females at 10 and 100 µM while permethrin showed a decrease of the activity at only 100 µM in females. These findings appear to support the association between permethrin and sex-specific epigenetic alteration (Blanc et al., 2021). The current study also found no cytotoxic effects after exposure to both compounds, contrary to (Shi et al., 2011), who suggest apoptosis inducted by cypermethrin and permethrin in the brain and spinal cord of larvae zebrafish. Their effects on Na^+ channels may explain the differences between neuronal activities of both types of pyrethroids. It is generally accepted that the main target of pyrethroids are voltage-gated sodium channels (VGSCs). Both chemicals are neurotoxic insecticides that allow Na^+ to flow into the cell, producing depolarization of the membrane potential. Type I and type II pyrethroids could have different effects because of their different molecular structure. While type I pyrethroids lack an α-cyano group, type II contains an α-cyano group and opens Na^+ channels for a longer time (Richardson et al., 2019). Type II exposure results in a prolonged effect on neurons, producing a depolarization block accompanied by suppression cellular excitability (Range et al., 2012). Therefore, type II pyrethroids may produce a more profound inhibition of the activity. Nevertheless, in several studies, the effects of pyrethroids were also associated with the opening voltage-gated calcium channels (VGCCs) and the inhibition of $GABA_A$ receptors (Johnstone et al., 2017b; Meyer et al., 2008). In addition, both pyrethroids may also likely be involved in the thyroid hormone agonist (Leemans et al., 2019). The differences between sex in permethrin might also be explained as differences in the expression of hormone receptors and may be related to the endocrine disruption effect. However, the hormone receptor was not demonstrated to affect the neuronal network activity since previous data showed that triiodothyronine (an agonist of TH receptor) and NH-3 (an antagonist of TH receptor) had no significant effect on neuronal activity

(**figures 12** and **13** of the appendix). There is, thereby an unclear knowledge about the mechanisms that produce the differences in neuronal activity.

Our results suggest that BBzP at the highest concentration (100 µM) produced almost full inhibition in both sexes. In good agreement with our results, previous data suggest that neuronal activity decreased completely after acute exposure to BBzP in males and females (see **figure 10** in the appendix). Further, we found no cytotoxic effects of BBzP. Thus, our results indicate neurotoxic effects after developmental exposure to the highest concentration of BBzP, without loss of cell viability. The decrease in activity could be attributed to the capacity of BBzP to bind hormone receptors and ion channel targets. Notably, previous studies suggested that BBzP might be related to the inhibition of the prostaglandin E2 (PGE2) receptor (Land et al., 2021), and it has also been associated with prostaglandin synthesis (Kristensen et al., 2011). Comparatively, acute exposition to PGE, using rat primary cortical cells measured by MEA recording suggested a small non-significative drop-in the neuronal activity at 0.1 µm (**figure 14** in the appendix). Surprisingly, BBzP might be linked also to the disruption in the thyroid homeostasis (Ishihara et al., 2003; Li et al., 2022) and the estrogenic and an antiandrogenic effects (Se & Byung, 2005; Swan et al., 2005). This fact suggests the possibility that sex-specific differences in our results are due to the disruption of estrogen receptors. Nonetheless, our data is insufficient to demonstrate that hormone disruption of BBzP may cause neurotoxicity since BBzP has been shown to bind as well as ligand-gated ion channels. Likewise, studies demonstrated the ability of BBzP to inhibit VGCCs, nicotinic acetylcholine receptors (nAChRs) and purigenic P2X receptors (P2XR), decreasing calcium influx. Neurons are excitable cells affected by the change in Ca^{2+} and fluctuations in Ca^{2+}influx affect normal neuronal activity. Thus, a calcium modulation reduction may decrease network neuronal activity (Liu & Chen, 2006; Shantanam et al., 2018; Shen & Yakel, 2009).

Interestingly, the expression of the hormone receptors implicated in the endocrine disrupting mechanism of the compounds used were studied before. (*Novel Testing Strategies for Endocrine Disruptors Linked to Developmental Neurotoxicity D2.1 Report on Involvement of Endocrine Pathways on DNT-Related KE in Vitro*, 2021). In this research, endocrine receptors gene expressions were analyzed over 28 days in vitro. Data concluded that Liver X/ oxysterols receptor beta (LXR) was presented in males on DIV 4-28 and in females on DIV 4, 7, 10, 14, 21, and 28. Estrogen receptor (ER) beta was expressed in males and females on DIV 0-28 and ER alpha was expressed in males on DIV 0-21 and in females on DIV 0-28. Thyroid hormone receptors alpha (THR) were expressed on DIV 0-14 in males and on DIV 0-21 in females. Thyroid hormone receptors beta were detected on DIV 4-28 in males and in DIV 4, 7, 14 and 21 in females. Prostaglandin E2 receptor (PGER) was expressed from DIV 0 to DIV 28 in both sexes. Consequently, these results indicate that the model used in our study allows investigating the effects of the compounds used on the hormone receptor expressed.

Detailed investigation of the effects of EDCs in neuronal network activity is a further step to identifying a potential direct role of EDCs in DNT. Although it is unclear what mechanisms are involved in the neurotoxic effect since chemicals used are shown to bind some targets with relevant

importance in the developmental brain, the data suggest that there are neuronal effects produced after exposure to chemicals. The underlying neurotoxic mechanism of these substances is still unexplained, and more studies are needed to gain insight into the EDC-induced DNT. For instance, it would be interesting to test the potential EDCs compounds with agonists and antagonists of the hormone receptor that they potentially bind and compare results with our current data.

In line with results from other authors, this study reveals the sensitivity and specificity of MEA and rat cortical cell culture as a reliable and feasible screening method for neurotoxicity testing (Hondebrink et al., 2016; McConnell et al., 2012; Shafer, 2019; Strickland et al., 2018). However, future experiments may apply a human in vitro model such as hiPSC-derived neuronal to avoid the interspecies problem. Besides, future research opportunities might be compared to gain insight into the differences between cultures.

Brain development in humans begins in the second -week post-conception and continues into young adulthood till 20 years (Tierney & Nelson, 2009). Nevertheless, in this study, we exposed the cells to compounds for 21 days since it is the maximum time that cell culture can be kept with neuronal activity (Gerber et al., 2021b). The time frame could be a limitation in the study of developmental effects. At DIV 0 rat cortical cell culture starts to develop neuronal networks. However, the exposure to compounds in our experiments starts at DIV 7 since the neuronal network is fully mature (Gerber et al., 2021b). To close the experiments to the real human life scenario, it may be beneficial to prolong the exposure time and start the exposure at DIV 0.

There are some limitations to the cell viability assay used in this study since this method measures the metabolic activity of the cells. Chemicals can produce cytotoxicity causing cell damage and/or cell death. Sometimes, cells damaged are alive and they may maintain mitochondrial activity. Moreover, neurons can compensate when other neurons die and there is also the possibility that cells stressed caused hyper metabolic activity. Consequently, future work is planned to assess an extra experiment to gain knowledge about the cytotoxicity of the compounds in the cells. Moreover, neurons can compensate when other neurons die and there is also the possibility that cells stressed caused hyper metabolic activity. Consequently, future work is planned to assess an extra experiment to gain knowledge about the cytotoxicity of the compounds in the cells. For instance, immunohistochemistry assay (IHC), provides an identification of neurons visualizing an antibody-antigen interaction (Ramos-Vara, 2005). This technique allows to identify and differentiate specific neurons and glial cell types in the cerebral cortex. Moreover, IHC is suitable for visualizing and defining the shape and sizes of the cells (García-Cabezas et al., 2016).

In summary, within the limitations of this study, the current findings can be evaluated as a useful empirical reference to gain more insights into the links between endocrine disruption and DNT. However, there is a set of suspected endocrine disruptor chemicals associated with human neuronal effects, and future research to gain a more comprehensive view is needed. There is an urgent need to test more chemicals to generate scientific knowledge in the gap between EDCs and DNT for risk assessment. Humans are continuously exposed to ECDs presented in food, air, water, and products that may negatively affect our health. Consequently, policies should promote public awareness and

risk communication regarding these chemicals and their effects on our health. Moreover, governments should improve regulations to minimize human exposure and protect public health from the risks of EDCs.

8. Acknowledge

I gratefully acknowledge the members of the Neurotoxicology Research Group for the assistance and the support. This work is part of the ENDpoiNTs project and it has received funding from the Faculty of Veterinary Medicine of Utrecht University and the European Union's Horizon 2020 research and innovation programme under grant agreement No 825759.

9. Appendix

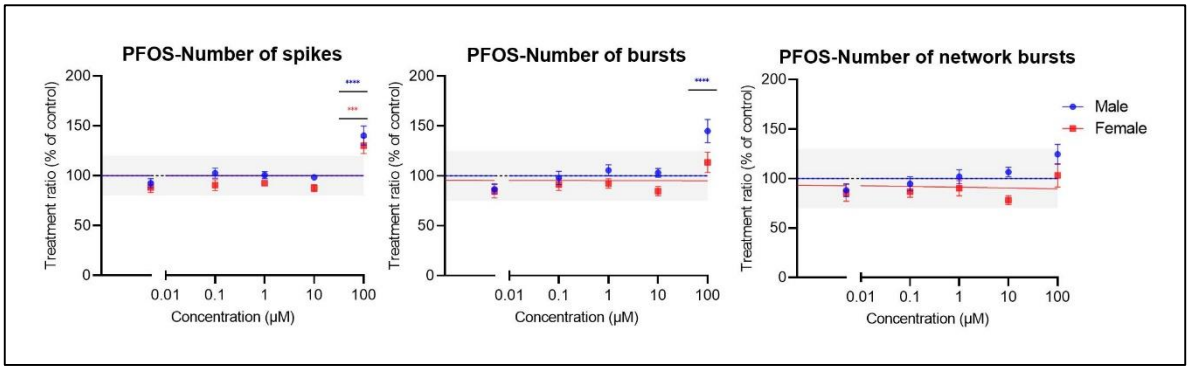


Figure 7. Acute effect of PFOS on neuronal activity of rat primary cortical cells. Concentration-response relationship for PFOS (0.01-100 µM) on neuronal network activity in rat cortical cells after 48 hours of exposure on the MEA. Neuronal activity is represented as the number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for females (red) and males (blue). Effects are depicted as mean treatment ratio normalized to control (DMSO set to 100%) ± SEM. The grey area presents a 30% benchmark response derived from the average variation in control experiments at baseline. Significant concentration- dependent effect is presented by $p<0.05$. Asterisks represent significant differences compared to the control (The color blue indicates the males and the red the females).

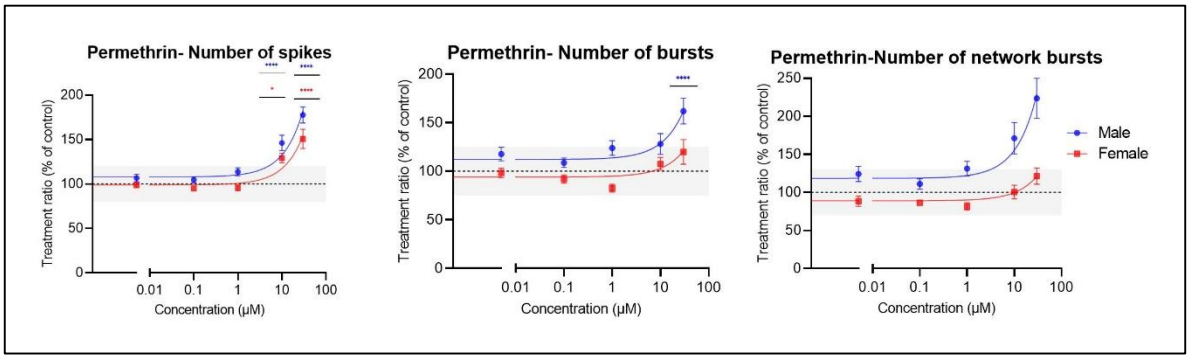


Figure 8. Acute effect of permethrin on neuronal activity of rat primary cortical cells. Concentration-response relationship for permethrin (0.01-100 µM) on neuronal network activity in rat cortical cells after 48 hours of exposure on the MEA. Neuronal activity is represented as the number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for females (red) and males (blue). Effects are depicted as mean treatment ratio normalized to control (DMSO set to 100%) ± SEM. The grey area presents a 30% benchmark response derived from the average variation in control experiments at baseline. Significant concentration- dependent effect is presented by $p<0.05$. Asterisks represent significant differences compared to the control. (The color blue indicates the males and the red the females).

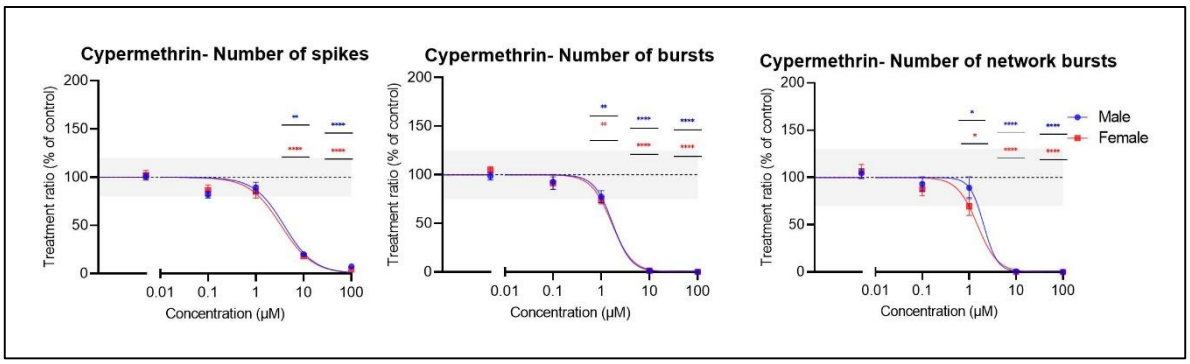


Figure 9. Acute effect of cypermethrin on neuronal activity of rat primary cortical cells. Concentration-response relationship for permethrin (0.01-100 µM) on neuronal network activity in rat cortical cells after 48 hours of exposure on the MEA. Neuronal activity is represented as the number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for females (red) and males (blue). Effects are depicted as mean treatment ratio normalized to control (DMSO set to 100%) ± SEM. The grey area presents a 30% benchmark response derived from the average variation in control experiments at baseline. Significant concentration- dependent effect is presented by $p<0.05$. Asterisks represent significant differences compared to the control. (The color blue indicates the males and the red the females)

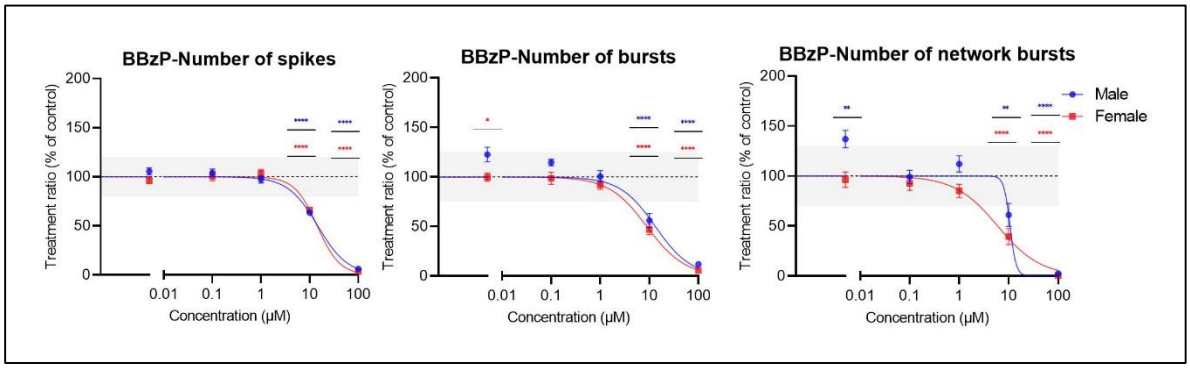


Figure 10. Acute effect of BBzpP on neuronal activity of rat primary cortical cells. Concentration-response relationship for BBzP (0.01-100 µM) on neuronal network activity in rat cortical cells after 48 hours of the exposure on the MEA. Neuronal activity is represented as the number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for females (red) and males (blue). Effects are depicted as mean treatment ratio normalized to control (DMSO set to 100%) ± SEM. The grey area presents a 30% benchmark response derived from the average variation in control experiments at baseline. Significant concentration- dependent effect is presented by $p<0.05$. Asterisks represent significant differences compared to the control. (The color blue indicates the males and the red the females)

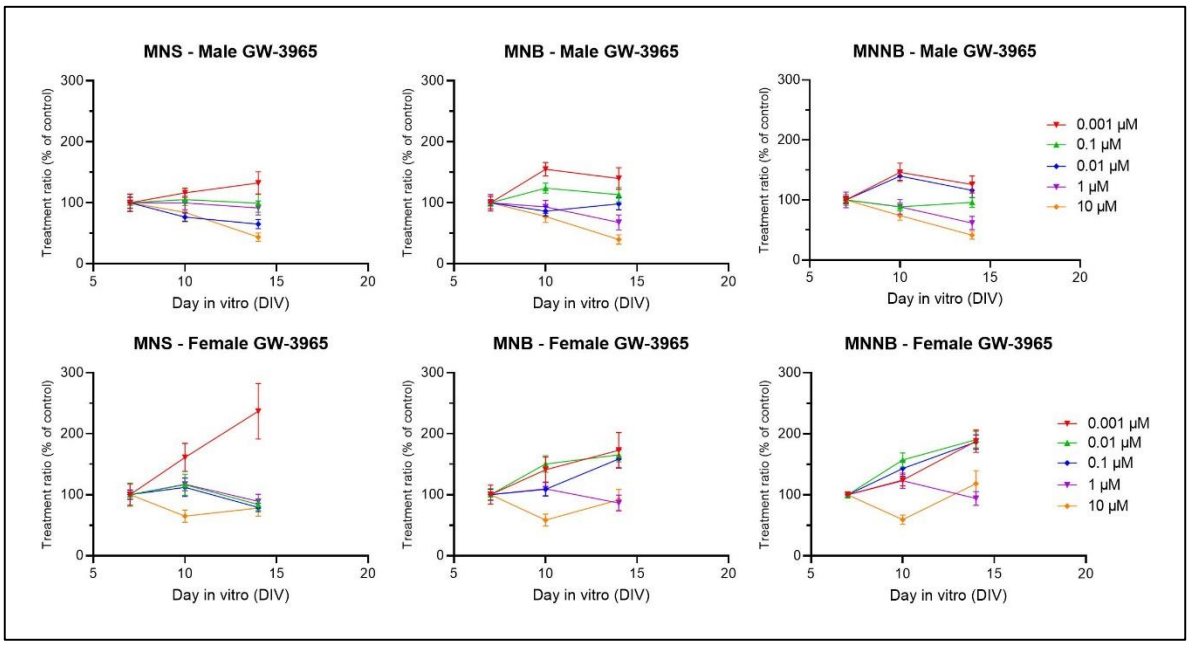


Figure 11. Developmental effects of LXR agonist (GW-3965), on neuronal activity of rat primary cortical cells. Concentration-response relationship for GW-3965 (0.001-10 µM) is tested over time during chronic (DIV 7-14) MEA experiments. Neuronal activity is represented as the number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for female (top graphs) and male (bottom graphs) cells. The results are presented as mean treatment ratio normalized to control (DMSO set to 100%) ± SEM. The color of the lines indicates the exposure concentration (red for 0,001 µM; green for 0.01 µM; blue for 0.1 µM; purple for 1 µM; yellow for 10 µM).

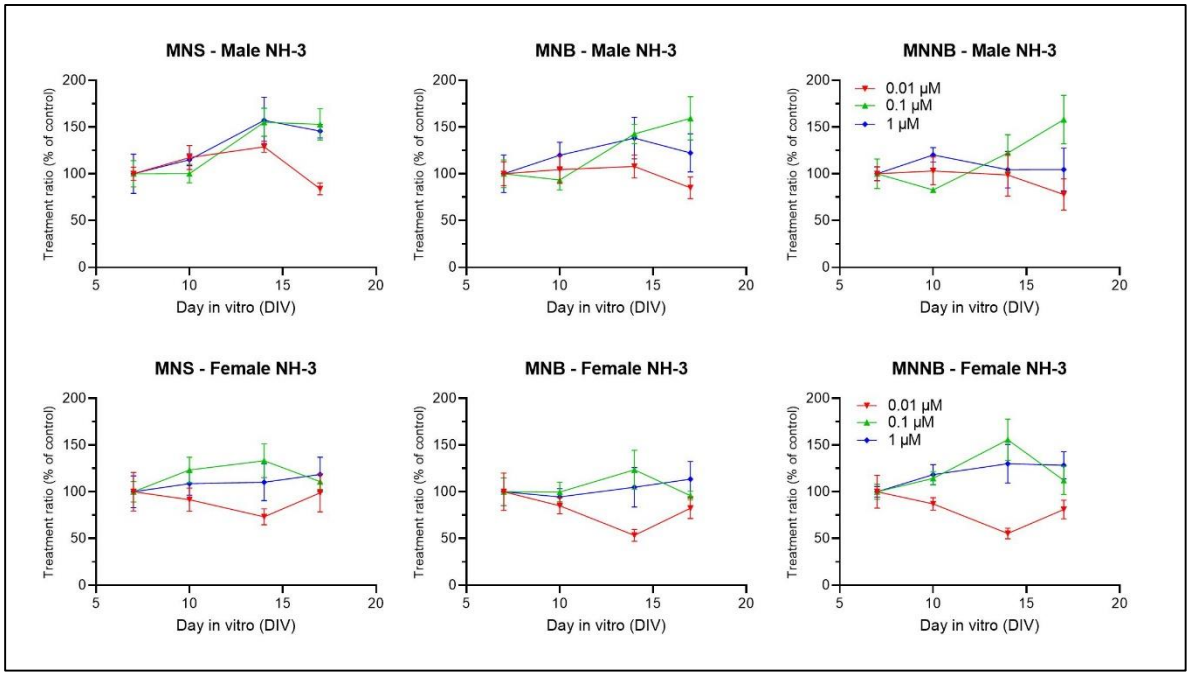


Figure 12. Developmental effects of thyroid hormone receptor antagonist (NH3) on neuronal activity of rat primary cortical cells. Concentration-response relationship for NH3 (0.01-1 µM) is tested over time during chronic (DIV 7-21) MEA experiments. Neuronal activity is represented as the number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for female (top graphs) and male (bottom graphs) cells. The results are presented as mean treatment ratio normalized to control (DMSO set to 100%) ± SEM. The color of the lines indicates the exposure concentration (red for 0,01 µM; green for 0.1 µM; blue for 1 µM).

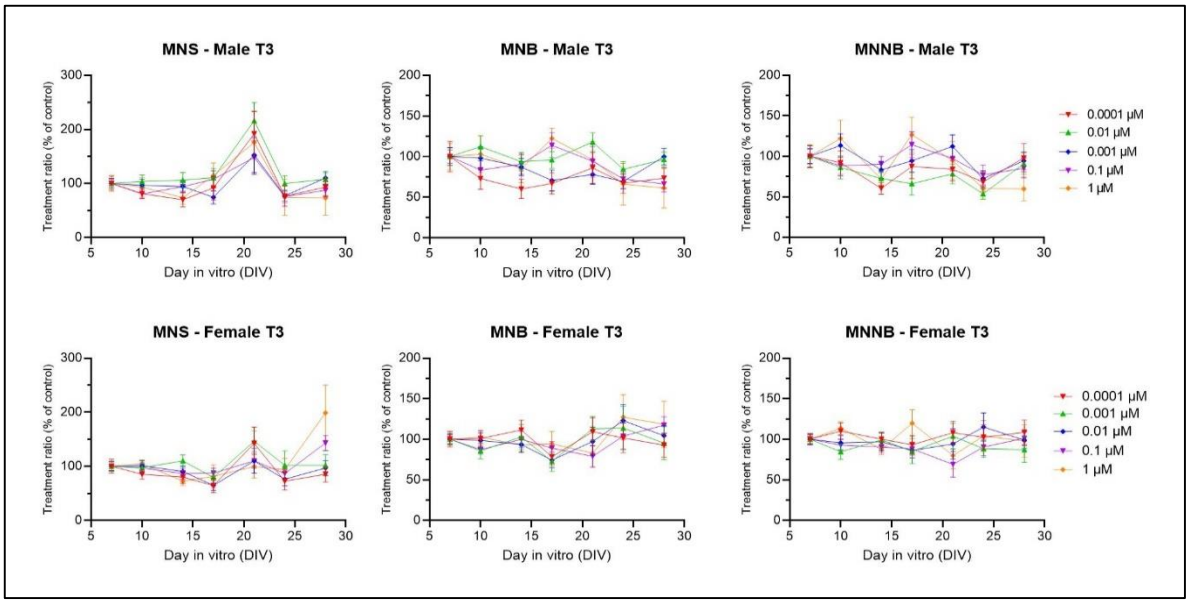


Figure 13. Developmental effects of a thyroid hormone receptor agonist (triiodothyronine, T3**) on neuronal activity of rat primary cortical cells.** Concentration-response relationship for triiodothyronine (0.0001-1 µM) is tested over time during chronic (DIV 7-21) MEA experiments. Neuronal activity is represented as the number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for female (top graphs) and male (bottom graphs) cells. The results are presented as mean treatment ratio normalized to control (DMSO set to 100%) ± SEM. The color of the lines indicates the exposure concentration (red for 0,0001 µM; green for 0.001 µM; blue for 0.01 µM; purple for 0.1 µM; yellow for 1 µM).

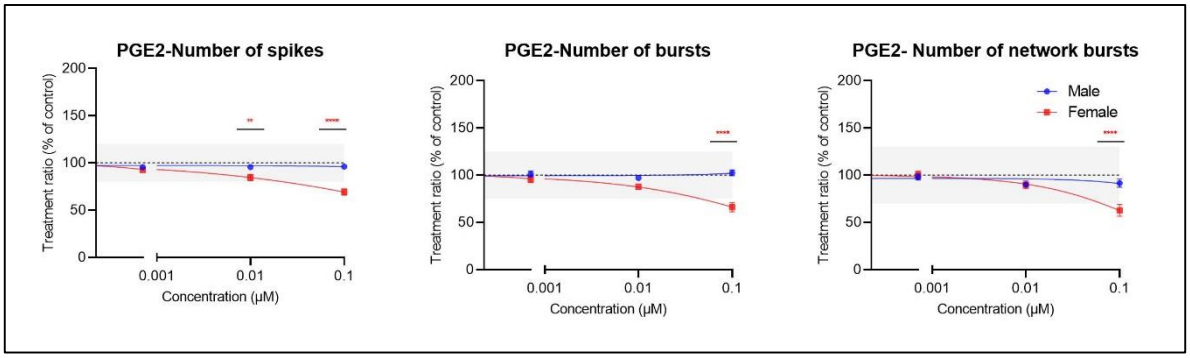


Figure 14. Acute effect of PGE2 receptor agonist on neuronal activity of rat primary cortical cells. Concentration-response relationship for PGE2 (0.001-0.1 µM) on neuronal network activity in rat cortical cells after 48 hours of the exposure on the MEA. Neuronal activity is represented as the number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for females (red) and males (blue). Effects are depicted as mean treatment ratio normalized to control (DMSO set to 100%) ± SEM. The grey area presents a 30% benchmark response derived from the average variation in control experiments at baseline. Significant concentration- dependent effect is presented by p<0.05.

Concentration	Significant differences MNS (p<0.05)		Significant differences MNB (p<0.05)		Significant differences MNNB (p<0.05)	
	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE
0.01 µM	DIV 28	DIV 28	DIV 17	DIV 28	DIV 14 DIV 28	-
0.1 µM	DIV 7	DIV 7	-	-	DIV 14	-
1 µM	-	-	-	-	-	-
10 µM	-	-	-	-	-	-
100 µM	DIV 10 DIV 14 DIV 17 DIV 21 DIV 24 DIV 28	DIV 10 DIV 14 DIV 21 DIV 24 DIV 28	DIV 10 DIV 14 DIV 17 DIV 21 DIV 24 DIV 28	DIV 10 DIV 21 DIV 24	DIV 10 DIV 21 DIV 24	DIV 10

Table 3. Statistical significant results after chronic exposure to BBzP. After data and statistical analysis, concentration and time response of neuronal activity were represented as number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for females and males. Concentration-response relationship for BBzP (0.01-100 µM) was assessed over time during chronic (DIV7-28). Results in MNS, MNB and MNNB were compared to the DMSO control and normalized to DIV 7. Data points included were those with a significant difference compared to the control p ≤0.05.

Concentration	Significant differences MNS (p<0.05)		Significant differences MNB (p<0.05)		Significant differences MNNB (p<0.05)	
	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE
0.01 µM	DIV 7	DIV 10	DIV 7 DIV 10	DIV 7 DIV 10	DIV 7 DIV 10	DIV 7 DIV 10
0.1 µM	-	DIV 10	-	-	-	-
1 µM	-	-	-	-	-	-
10 µM	DIV 10 DIV 17 DIV 24 DIV 28	DIV 14 DIV 17 DIV 24 DIV 28	DIV 7 DIV 10 DIV 17 DIV 24 DIV 28	DIV 10 DIV 14 DIV 17 DIV 24 DIV 28	DIV 10 DIV 17 DIV 21 DIV 24 DIV 28	DIV 14 DIV 17 DIV 24 DIV 28
100 µM	DIV 7 DIV 10 DIV 17 DIV 24 DIV 28	DIV 7 DIV 14 DIV 17 DIV 21 DIV 24 DIV 28	DIV 7 DIV 10 DIV 17 DIV 21 DIV 24 DIV 28	DIV 7 DIV 10 DIV 14 DIV 17 DIV 24 DIV 28	DIV 7 DIV 10 DIV 17 DIV 21 DIV 24 DIV 28	DIV 7 DIV 10 DIV 17 DIV 21 DIV 24 DIV 28

Table 4. Statistical significant results after chronic exposure to cypermethrin. After data and statistical analysis, concentration and time response of neuronal activity were represented as number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for females and males. Concentration-response relationship for cypermethrin (0.01-100 µM) was assessed over time during chronic (DIV7-28). Results in MNS, MNB and MNNB were compared to the DMSO control and normalized to DIV 7. Data points included were those with a significant difference compared to the control $p \leq 0.05$.

Concentration	Significant differences MNS (p<0.05)		Significant differences MNB (p<0.05)		Significant differences MNNB (p<0.05)	
	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE
0.01 µM	DIV 7 DIV 24 DIV 28	DIV 17	DIV 28	DIV 10 DIV 24	DIV 28	DIV 17 DIV 21
0.1 µM	DIV 28	-	-	DIV 10	-	-
1 µM	-	-	-	-	-	-
10 µM	DIV 28		DIV 10	DIV 17 DIV 21	DIV 10 DIV 17 DIV 24 DIV 28	DIV 17 DIV 21 DIV 24 DIV 28
100 µM	DIV 7 DIV 10	DIV 14 DIV 17 DIV 21 DIV 24 DIV 28	DIV 7	DIV 14 DIV 17 DIV 21 DIV 28	-	DIV 21 DIV 28

Table 5. Statistical significant results after chronic exposure to permethrin. After data and statistical analysis, concentration and time response of neuronal activity were represented as number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for females and males. Concentration-response relationship for permethrin (0.01-100 µM) was assessed over time during chronic (DIV7-28). Results in MNS, MNB and MNNB were compared to the DMSO control and normalized to DIV 7. Data points included were those with a significant difference compared to the control p ≤0.05.

Concentration	Significant differences MNS (p<0.05)		Significant differences MNB (p<0.05)		Significant differences MNNB (p<0.05)	
	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE
0.01 µM	DIV 24	DIV 10	DIV 14	-	-	DIV 10
0.1 µM	-	DIV 24	-	-	-	-
1 µM	-	-	-	-	-	-
10 µM	-	-	-	-	-	-
100 µM	-	DIV 7 DIV 17 DIV 21 DIV 24 DIV 28	-	DIV 7	DIV 21	-

Table 6. Statistical significant results after chronic exposure to PFOS. After data and statistical analysis, concentration and time response of neuronal activity were represented as number of spikes (MNS), bursts (MNB), and network bursts (MNNB) for females and males. Concentration-response relationship for PFOS (0.01-100 µM) was assessed over time during chronic (DIV7-28). Results in MNS, MNB and MNNB were compared to the DMSO control and normalized to DIV 7. Data points included were those with a significant difference compared to the control p ≤0.05.

10. References

Andersson, N., Arena, M., Auteri, D., Barmaz, S., Grignard, E., Kienzler, A., Lepper, P., Lostia, A. M., Munn, S., Morte, J. M. P., Pellizzato, F., Tarazona, J., Terron, A., & der Linden, S. Van. (2018). Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009. *EFSA Journal*, *16*(6). https://doi.org/10.2903/j.efsa.2018.5311

Báez-Becerra, C., Filipello, F., Sandoval-Hernández, A., Arboleda, H., & Arboleda, G. (2018). Liver X Receptor Agonist GW3965 Regulates Synaptic Function upon Amyloid Beta Exposure in Hippocampal Neurons. *Neurotoxicity Research*, *33*(3), 569–579. https://doi.org/10.1007/s12640-017-9845-3

Berntsen, H. F., Bjørklund, C. G., Strandabø, R., Haug, T. M., Moldes-Anaya, A., Fuentes-Lazaro, J., Verhaegen, S., Paulsen, R. E., Tasker, R. A., & Ropstad, E. (2018). PFOS-induced excitotoxicity is dependent on Ca 2+ influx via NMDA receptors in rat cerebellar granule neurons. *Toxicology and Applied Pharmacology*, *357*(June), 19–32. https://doi.org/10.1016/j.taap.2018.08.015

Blanc, M., Antczak, P., Cousin, X., Grunau, C., Scherbak, N., Rüegg, J., & Keiter, S. H. (2021). The insecticide permethrin induces transgenerational behavioral changes linked to transcriptomic and epigenetic alterations in zebrafish (Danio rerio). *Science of the Total Environment*, *779*. https://doi.org/10.1016/j.scitotenv.2021.146404

Cao, Y., & Ng, C. (2021). Absorption, distribution, and toxicity of per- And polyfluoroalkyl substances (PFAS) in the brain: A review. In *Environmental Science: Processes and Impacts* (Vol. 23, Issue 11, pp. 1623–1640). Royal Society of Chemistry. https://doi.org/10.1039/d1em00228g

Cohn, B. A., La Merrill, M., Krigbaum, N. Y., Yeh, G., Park, J. S., Zimmermann, L., & Cirillo, P. M. (2015). DDT exposure in utero and breast cancer. *Journal of Clinical Endocrinology and Metabolism*, *100*(8), 2865–2872. https://doi.org/10.1210/jc.2015-1841

Combarnous, Y. (2017). Endocrine Disruptor Compounds (EDCs) and agriculture: The case of pesticides. *Comptes Rendus - Biologies*, *340*(9–10), 406–409. https://doi.org/10.1016/j.crvi.2017.07.009

Cui, L., Zhou, Q. F., Liao, C. Y., Fu, J. J., & Jiang, G. Bin. (2009). Studies on the toxicological effects of PFOA and PFOS on rats using histological observation and chemical analysis. *Archives of Environmental Contamination and Toxicology*, *56*(2), 338–349. https://doi.org/10.1007/s00244-008-9194-6

European Parliament. (2003). *REGULATION (EC) No 304/2003 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 28 January 2003 concerning the export and import of dangerous chemicals*. *304*, 1–34.

Fàbrega, F., Kumar, V., Schuhmacher, M., Domingo, J. L., & Nadal, M. (2014). PBPK modeling for PFOS and PFOA: Validation with human experimental data. *Toxicology Letters*, *230*(2), 244–251. https://doi.org/10.1016/j.toxlet.2014.01.007

Gaballah, S., Swank, A., Sobus, J. R., Howey, X. M., Schmid, J., Catron, T., McCord, J., Hines, E., Strynar, M., & Tal, T. (2020). Evaluation of developmental toxicity, developmental neurotoxicity, and tissue dose in zebrafish exposed to genX and other PFAS. *Environmental Health*

Perspectives, *128*(4). https://doi.org/10.1289/EHP5843

García-Cabezas, M., John, Y. J., Barbas, H., & Zikopoulos, B. (2016). Distinction of neurons, glia and endothelial cells in the cerebral cortex: An algorithm based on cytological features. *Frontiers in Neuroanatomy*, *10*(NOV), 1–28. https://doi.org/10.3389/fnana.2016.00107

Gerber, L. S., van Melis, L. V. J., van Kleef, R. G. D. M., de Groot, A., & Westerink, R. H. S. (2021a). Culture of Rat Primary Cortical Cells for Microelectrode Array (MEA) Recordings to Screen for Acute and Developmental Neurotoxicity. *Current Protocols*, *1*(6). https://doi.org/10.1002/cpz1.158

Gerber, L. S., van Melis, L. V. J., van Kleef, R. G. D. M., de Groot, A., & Westerink, R. H. S. (2021b). Culture of Rat Primary Cortical Cells for Microelectrode Array (MEA) Recordings to Screen for Acute and Developmental Neurotoxicity. *Current Protocols*, *1*(6), 1–35. https://doi.org/10.1002/cpz1.158

Gilbert, M. E., Rovet, J., Chen, Z., & Koibuchi, N. (2012). Developmental thyroid hormone disruption: Prevalence, environmental contaminants and neurodevelopmental consequences. *NeuroToxicology*, *33*(4), 842–852. https://doi.org/10.1016/j.neuro.2011.11.005

Heberden, C. (2017). Sex steroids and neurogenesis. *Biochemical Pharmacology*, *141*, 56–62. https://doi.org/10.1016/j.bcp.2017.05.019

Hill, R. A., Wu, Y. W. C., Kwek, P., & Van den Buuse, M. (2012). Modulatory Effects of Sex Steroid Hormones on Brain-Derived Neurotrophic Factor-Tyrosine Kinase B Expression during Adolescent Development in C57Bl/6 Mice. *Journal of Neuroendocrinology*, *24*(5), 774–788. https://doi.org/10.1111/j.1365-2826.2012.02277.x

Högberg, J., Hanberg, A., Berglund, M., Skerfving, S., Remberger, M., Calafat, A. M., Filipsson, A. F., Jansson, B., Johansson, N., Appelgren, M., & Håkansson, H. (2008). Phthalate diesters and their metabolites in human breast milk, blood or serum, and urine as biomarkers of exposure in vulnerable populations. *Environmental Health Perspectives*, *116*(3), 334–339. https://doi.org/10.1289/ehp.10788

Hondebrink, L., Verboven, A. H. A., Drega, W. S., Schmeink, S., de Groot, M. W. G. D. M., van Kleef, R. G. D. M., Wijnolts, F. M. J., de Groot, A., Meulenbelt, J., & Westerink, R. H. S. (2016). Neurotoxicity screening of (illicit) drugs using novel methods for analysis of microelectrode array (MEA) recordings. *NeuroToxicology*, *55*, 1–9. https://doi.org/10.1016/j.neuro.2016.04.020

Ishihara, A., Nishiyama, N., Sugiyama, S. I., & Yamauchi, K. (2003). The effect of endocrine disrupting chemicals on thyroid hormone binding to Japanese quail transthyretin and thyroid hormone receptor. *General and Comparative Endocrinology*, *134*(1), 36–43. https://doi.org/10.1016/S0016-6480(03)00197-7

Johnstone, A. F. M., Strickland, J. D., Crofton, K. M., Gennings, C., & Shafer, T. J. (2017a). Effects of an environmentally-relevant mixture of pyrethroid insecticides on spontaneous activity in primary cortical networks on microelectrode arrays. *NeuroToxicology*, *60*, 234–239. https://doi.org/10.1016/j.neuro.2016.05.005

Kosnik, M. B., Strickland, J. D., Marvel, S. W., Wallis, D. J., Wallace, K., Richard, A. M., Reif, D. M., & Shafer, T. J. (2020). Concentration–response evaluation of ToxCast compounds for

multivariate activity patterns of neural network function. *Archives of Toxicology*, *94*(2), 469–484. https://doi.org/10.1007/s00204-019-02636-x

Kristensen, D. M., Skalkam, M. L., Audouze, K., Lesné, L., Desdoits-Lethimonier, C., Frederiksen, H., Brunak, S., Skakkebaek, N. E., Jégou, B., Hansen, J. B., Junker, S., & Leffers, H. (2011). Many putative endocrine disruptors inhibit prostaglandin synthesis. *Environmental Health Perspectives*, *119*(4), 534–541. https://doi.org/10.1289/ehp.1002635

Kunno, J., Ong-Artborirak, P., Taneepanichskul, N., Robson, M. G., & Siriwong, W. (2021). Effect of pyrethroid insecticides exposure in relation to pyrethroid metabolite and GABA concentration of young children, Bangkok Thailand. *Human and Ecological Risk Assessment*, *27*(1), 1–14. https://doi.org/10.1080/10807039.2019.1689098

Land, K. L., Lane, M. E., Fugate, A. C., & Hannon, P. R. (2021). Ovulation is inhibited by an environmentally relevant phthalate mixture in mouse antral follicles in vitro. *Toxicological Sciences*, *179*(2), 195–205. https://doi.org/10.1093/toxsci/kfaa170

Leemans, M., Couderq, S., Demeneix, B., & Fini, J. B. (2019). Pesticides With Potential Thyroid Hormone-Disrupting Effects: A Review of Recent Data. In *Frontiers in Endocrinology* (Vol. 10). Frontiers Media S.A. https://doi.org/10.3389/fendo.2019.00743

Lenters, V., Iszatt, N., Forns, J., Čechová, E., Kočan, A., Legler, J., Leonards, P., Stigum, H., & Eggesbø, M. (2019). Early-life exposure to persistent organic pollutants (OCPs, PBDEs, PCBs, PFASs) and attention-deficit/hyperactivity disorder: A multi-pollutant analysis of a Norwegian birth cohort. *Environment International*, *125*(April 2018), 33–42. https://doi.org/10.1016/j.envint.2019.01.020

Li, J., Xu, Y., Jiang, Y., Li, N., Li, Z., Kong, D., Guo, X., Zhang, J., & Zuo, R. (2022). Nongenomic effects and mechanistic study of butyl benzyl phthalate-induced thyroid disruption: Based on integrated in vitro, in silico assays and proteome analysis. *Science of the Total Environment*, *836*(March). https://doi.org/10.1016/j.scitotenv.2022.155715

Liu, P. S., & Chen, Y. Y. (2006). Butyl benzyl phthalate blocks Ca2+ signaling coupled with purinoceptor in rat PC12 cells. *Toxicology and Applied Pharmacology*, *210*(1–2), 136–141. https://doi.org/10.1016/j.taap.2005.09.012

Ludwicki, J. K., Góralczyk, K., Struciński, P., Wojtyniak, B., Rabczenko, D., Toft, G., Lindh, C. H., Jönsson, B. A. G., Lenters, V., Heederik, D., Czaja, K., Hernik, A., Pedersen, H. S., Zvyezday, V., & Bonde, J. P. (2015). Hazard quotient profiles used as a risk assessment tool for PFOS and PFOA serum levels in three distinctive European populations. *Environment International*, *74*, 112–118. https://doi.org/10.1016/j.envint.2014.10.001

Lupu, D., Andersson, P., Bornehag, C. G., Demeneix, B., Fritsche, E., Gennings, C., Lichtensteiger, W., Leist, M., Leonards, P. E. G., Ponsonby, A. L., Scholze, M., Testa, G., Tresguerres, J. A. F., Westerink, R. H. S., Zalc, B., & Rüegg, J. (2020). The endpoints project: Novel testing strategies for endocrine disruptors linked to developmental neurotoxicity. *International Journal of Molecular Sciences*, *21*(11). https://doi.org/10.3390/ijms21113978

McConnell, E. R., McClain, M. A., Ross, J., LeFew, W. R., & Shafer, T. J. (2012). Evaluation of multi-well microelectrode arrays for neurotoxicity screening using a chemical training set. *NeuroToxicology*, *33*(5), 1048–1057. https://doi.org/10.1016/j.neuro.2012.05.001

McEwen, B. S., & Milner, T. A. (2017). Understanding the broad influence of sex hormones and sex differences in the brain. *Journal of Neuroscience Research*, *95*(1–2), 24–39. https://doi.org/10.1002/jnr.23809

Meyer, D. A., Carter, J. M., Johnstone, A. F. M., & Shafer, T. J. (2008). Pyrethroid modulation of spontaneous neuronal excitability and neurotransmission in hippocampal neurons in culture. *NeuroToxicology*, *29*(2), 213–225. https://doi.org/10.1016/j.neuro.2007.11.005

Moog, N. K., Entringer, S., Heim, C., Wadhwa, P. D., Kathmann, N., & Buss, C. (2017). Influence of maternal thyroid hormones during gestation on fetal brain development. *Neuroscience*, *342*, 68–100. https://doi.org/10.1016/j.neuroscience.2015.09.070

Mouzat, K., Chudinova, A., Polge, A., Kantar, J., Camu, W., Raoul, C., & Lumbroso, S. (2019). Regulation of brain cholesterol: What role do liver X receptors play in neurodegenerative diseases? *International Journal of Molecular Sciences*, *20*(16). https://doi.org/10.3390/ijms20163858

Mughal, B. B., Fini, J. B., & Demeneix, B. A. (2018). Thyroid-disrupting chemicals and brain development: An update. *Endocrine Connections*, *7*(4), R160–R186. https://doi.org/10.1530/EC-18-0029

Novel Testing Strategies for Endocrine Disruptors linked to Developmental Neurotoxicity D2.1 Report on involvement of endocrine pathways on DNT-related KE in vitro. (2021).

Padayachee, K., Reynolds, C., Mateo, R., & Amar, A. (2023). A global review of the temporal and spatial patterns of DDT and dieldrin monitoring in raptors. *Science of the Total Environment*, *858*(October 2022), 159734. https://doi.org/10.1016/j.scitotenv.2022.159734

Pierozan, P., & Karlsson, O. (2021). Differential susceptibility of rat primary neurons and neural stem cells to PFOS and PFOA toxicity. *Toxicology Letters*, *349*, 61–68. https://doi.org/10.1016/j.toxlet.2021.06.004

Qian, Y., Shao, H., Ying, X., Huang, W., & Hua, Y. (2020). The Endocrine Disruption of Prenatal Phthalate Exposure in Mother and Offspring. *Frontiers in Public Health*, *8*(August). https://doi.org/10.3389/fpubh.2020.00366

Quindroit, P., Beaudouin, R., & Brochot, C. (2019). Estimating the cumulative human exposures to pyrethroids by combined multi-route PBPK models: Application to the French population. *Toxicology Letters*, *312*, 125–138. https://doi.org/10.1016/j.toxlet.2019.05.007

Ramos-Vara, J. A. (2005). Technical aspects of immunohistochemistry. *Veterinary Pathology*, *42*(4), 405–426. https://doi.org/10.1354/vp.42-4-405

Range, K., M, D., & Moser, Y. A. (2012). 基因的改变NIH Public Access. *Bone*, *23*(1), 1–7. https://doi.org/10.1016/j.taap.2008.10.006.Molecular

Richardson, J. R., Fitsanakis, V., Westerink, R. H. S., & Kanthasamy, A. G. (2019). Neurotoxicity of pesticides. *Acta Neuropathologica*, *138*(3), 343–362. https://doi.org/10.1007/s00401-019-02033-9

Sarigiannis, D. (2019). *Report on estimation of relevant tissue and biological fluid doses of 1st set of priority compounds in the EU population using refined PBTK modelling Deliverable Report WP12 - From HBM to exposure Deadline : December 2018 Upload by Coordinator : 12 August*

2019. *733032*.

Se, C. K., & Byung, M. L. (2005). DNA methylation of estrogen receptor α gene by phthalates. *Journal of Toxicology and Environmental Health - Part A*, *68*(23–24), 1995–2003. https://doi.org/10.1080/15287390491008913

Shafer, T. J. (2019). Application of Microelectrode Array Approaches to Neurotoxicity Testing and Screening BT - In Vitro Neuronal Networks: From Culturing Methods to Neuro-Technological Applications. *In Vitro Neuronal Networks. Advances in Neurobiology*, 275–297. https://doi.org/10.1007/978-3-030-11135-9_12

Shafer, T. J., Meyer, D. A., & Crofton, K. M. (2005). Developmental neurotoxicity of pyrethroid insecticides: Critical review and future research needs. In *Environmental Health Perspectives* (Vol. 113, Issue 2, pp. 123–136). https://doi.org/10.1289/ehp.7254

Shantanam, S., MUELLER, Stiles, J., & Jernigan, T. L. (2018). 乳鼠心肌提取 HHS Public Access. *Physiology & Behavior*, *20*(1), 327–348. https://doi.org/10.1007/s11065-010-9148-4

Shen, J. X., & Yakel, J. L. (2009). Nicotinic acetylcholine receptor-mediated calcium signaling in the nervous system. *Acta Pharmacologica Sinica*, *30*(6), 673–680. https://doi.org/10.1038/aps.2009.64

Shi, X., Gu, A., Ji, G., Li, Y., Di, J., Jin, J., Hu, F., Long, Y., Xia, Y., Lu, C., Song, L., Wang, S., & Wang, X. (2011). Developmental toxicity of cypermethrin in embryo-larval stages of zebrafish. *Chemosphere*, *85*(6), 1010–1016. https://doi.org/10.1016/j.chemosphere.2011.07.024

Simaremare, S. R. S., Hung, C. C., Hsieh, C. J., & Yiin, L. M. (2020). Relationship between organophosphate and pyrethroid insecticides in blood and their metabolites in urine: A pilot study. *International Journal of Environmental Research and Public Health*, *17*(1). https://doi.org/10.3390/ijerph17010034

Song, X., Wu, W., Warner, M., & Gustafsson, J. A. (2022). Liver X Receptor Regulation of Glial Cell Functions in the CNS. *Biomedicines*, *10*(9), 0–10. https://doi.org/10.3390/biomedicines10092165

Spulber, S., Kilian, P., Ibrahim, W. N. W., Onishchenko, N., Ulhaq, M., Norrgren, L., Negri, S., Tuccio, M. Di, & Ceccatelli, S. (2014). PFOS induces behavioral alterations, including spontaneous hyperactivity that is corrected by dexamfetamine in zebrafish larvae. *PLoS ONE*, *9*(4). https://doi.org/10.1371/journal.pone.0094227

Street, M. E., Angelini, S., Bernasconi, S., Burgio, E., Cassio, A., Catellani, C., Cirillo, F., Deodati, A., Fabbrizi, E., Fanos, V., Gargano, G., Grossi, E., Iughetti, L., Lazzeroni, P., Mantovani, A., Migliore, L., Palanza, P., Panzica, G., Papini, A. M., … Amarri, S. (2018). Current knowledge on endocrine disrupting chemicals (EDCs) from animal biology to humans, from pregnancy to adulthood: Highlights from a national italian meeting. *International Journal of Molecular Sciences*, *19*(6). https://doi.org/10.3390/ijms19061647

Strickland, J. D., Martin, M. T., Richard, A. M., Houck, K. A., & Shafer, T. J. (2018). Screening the ToxCast phase II libraries for alterations in network function using cortical neurons grown on multi-well microelectrode array (mwMEA) plates. *Archives of Toxicology*, *92*(1), 487–500. https://doi.org/10.1007/s00204-017-2035-5

Sundukov, Y. N. (2006). First record of the ground beetle Trechoblemus postilenatus (Coleoptera, Carabidae) in Primorskii krai. *Far Eastern Entomologist*, *165*(April), 16. https://doi.org/10.1002/tox

Swan, S. H., Main, K. M., Liu, F., Stewart, S. L., Kruse, R. L., Calafat, A. M., Mao, C. S., Redmon, J. B., Ternand, C. L., Sullivan, S., Teague, J. L., Drobnis, E. Z., Carter, B. S., Kelly, D., Simmons, T. M., Wang, C., Lumbreras, L., Villanueva, S., Diaz-Romero, M., … Maifeld, M. (2005). Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environmental Health Perspectives*, *113*(8), 1056–1061. https://doi.org/10.1289/ehp.8100

Tierney, A. L., & Nelson, C. A. (2009). Brain Development and the Role of Experience in the Early Years. *Zero to Three*, *30*(2), 9–13. http://www.ncbi.nlm.nih.gov/pubmed/23894221%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3722610

Trunnelle, K. J., Bennett, D. H., Tancredi, D. J., Gee, S. J., Stoecklin-Marois, M. T., Hennessy-Burt, T. E., Hammock, B. D., & Schenker, M. B. (2013). Pyrethroids in house dust from the homes of farm worker families in the MICASA study. *Environment International*, *61*, 57–63. https://doi.org/10.1016/j.envint.2013.09.007

Tukker, A. M., Bouwman, L. M. S., van Kleef, R. G. D. M., Hendriks, H. S., Legler, J., & Westerink, R. H. S. (2020). Perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) acutely affect human α1β2γ2L GABAA receptor and spontaneous neuronal network function in vitro. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-62152-2

Tukker, A. M., Wijnolts, F. M. J., de Groot, A., & Westerink, R. H. S. (2018). Human iPSC-derived neuronal models for in vitro neurotoxicity assessment. *NeuroToxicology*, *67*(April), 215–225. https://doi.org/10.1016/j.neuro.2018.06.007

Tukker, A. M., Wijnolts, F. M. J., de Groot, A., & Westerink, R. H. S. (2020). Applicability of hipsc-derived neuronal cocultures and rodent primary cortical cultures for in vitro seizure liability assessment. *Toxicological Sciences*, *178*(1), 71–87. https://doi.org/10.1093/toxsci/kfaa136

Welch, C., Johnson, E., Tupikova, A., Anderson, J., Tinsley, B., Newman, J., Widman, E., Alfareh, A., Davis, A., Rodriguez, L., Visger, C., Miller-Schulze, J. P., Lee, W., & Mulligan, K. (2022). Bisphenol a affects neurodevelopmental gene expression, cognitive function, and neuromuscular synaptic morphology in Drosophila melanogaster. *NeuroToxicology*, *89*, 67–78. https://doi.org/10.1016/j.neuro.2022.01.006

Woodward, A. R., Jennings, M. L., Percival, H. F., & Moore, C. T. (1993). Low clutch viability of American alligators on Lake Apopka. *Fla. Sci.*, *56*(1), 52–63.

Xie, S., Wang, T., Liu, S., Jones, K. C., Sweetman, A. J., & Lu, Y. (2013). Industrial source identification and emission estimation of perfluorooctane sulfonate in China. *Environment International*, *52*, 1–8. https://doi.org/10.1016/j.envint.2012.11.004

Xu, S., Zhang, H., Pao, P. C., Lee, A., Wang, J., Suen Chan, Y., Manno, F. A. M., Wan Chan, S., Han Cheng, S., & Chen, X. (2020). Exposure to phthalates impaired neurodevelopment through estrogenic effects and induced DNA damage in neurons. *Aquatic Toxicology*, *222*(December 2019), 105469. https://doi.org/10.1016/j.aquatox.2020.105469

Zhang, Q., Chen, X. Z., Huang, X., Wang, M., & Wu, J. (2019). The association between prenatal exposure to phthalates and cognition and neurobehavior of children-evidence from birth cohorts. *NeuroToxicology*, *73*(August 2018), 199–212. https://doi.org/10.1016/j.neuro.2019.04.007