DevTox

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Joint Research Centre

Adverse Outcome Pathways (AOPs)-driven evaluation of developmental neurotoxicity (DNT) induced by mixture of environmental chemicals

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Consensus statement on the need for innovation, transition and implementation of developmental neurotoxicity (DNT) testing for regulatory purposes

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ABSTRACT

This consensus statement voices the agreement of scientific stakeholders from regulatory agencies, academia and industry that a new framework needs adopting for assessment of chemicals with the potential to disrupt brain development. An increased prevalence of neurodevelopmental disorders in children has been observed that cannot solely be explained by genetics and recently pre- and postnatal exposure to environmental chemicals has been suspected as a causal factor. There is only very limited information on neurodevelopmental toxicity, leaving thousands of chemicals, that are present in the environment, with high uncertainty concerning their developmental neurotoxicity (DNT) potential. Closing this data gap with the current test guideline approach is not feasible, because the *in vivo* bioassays are far too resource-intensive concerning time, money and number of animals. A variety of *in vitro* methods are now available, that have the potential to close this data gap by permitting mode-of-action-based DNT testing employing human stem cells-derived neuronal/glial models. *In*

23 co-authors signed this Consensus Statement including academic scientists, EFSA, OECD, US EPA, BfR Germany, CAAT US/Europe, Danish EPA, SCHAT, Health Canada, JRC, BASF etc.

Perspectives | Brief Communication

Project TENDR: Targeting Environmental Neuro-Developmental Risks. The TENDR Consensus Statement

http://dx.doi.org/10.1289/EHP358

SUMMARY: Children in America today are at an unacceptably high risk of developing neurodevelopmental disorders that affect the brain and nervous system including autism, attention deficit hyperactivity disorder, intellectual disabilities, and other learning and behavioral disabilities.

CONCLUSION: Based on these findings, we assert that the current system in the United States for evaluating scientific evidence and making health-based decisions about environmental chemicals is fundamentally broken. To help reduce the unacceptably high prevalence of neurodevelopmental disorders in our children, we must eliminate or significantly reduce exposures to chemicals that contribute to these conditions. We must adopt a new framework for assessing chemicals that have the potential to disrupt brain development and prevent the use of those that may pose a risk. This consensus statement lays the foundation for developing recommendations to monitor, assess, and reduce exposures to neurotoxic chemicals. These measures are urgently needed if we are to protect healthy brain development so that current and future generations can reach their fullest potential.

47 USA scientists signed this statement.

(EHP, 2016, 124: 118-122)

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Humans, including the unborn, infants and children are indisputably co-exposed to more than one chemical at a time

Breast milk has been found to contain chemicals regulated as:

- **Cosmetics** including:
  - UV filters & musk fragrances
  - Parabens
  - Phthalate metabolites
- **POPs**
  - **pesticides** (chlorpyrifos-ethyl, chlordane, DDTs/DDEs/DDDs, HCHs, dieldrin, hexachlorobenzene, parlars
  - **PCBs** (28, 52,101, 118, 138,153, 180 etc.)
  - **PBDEs** (28, 47, 99, 100, 153, 154 etc)

(Schlumpf et al., 2010, Chemosphere, 81:1171–1183)

Mixture risk assessment (MRA): the cumulative risk to human health or the environment from **multiple chemicals** via **multiple routes**

- **Mixture effects'** are defined as either **an effect greater than the most potent single chemical** in the mixture, or an effect that is **additive or synergistic**

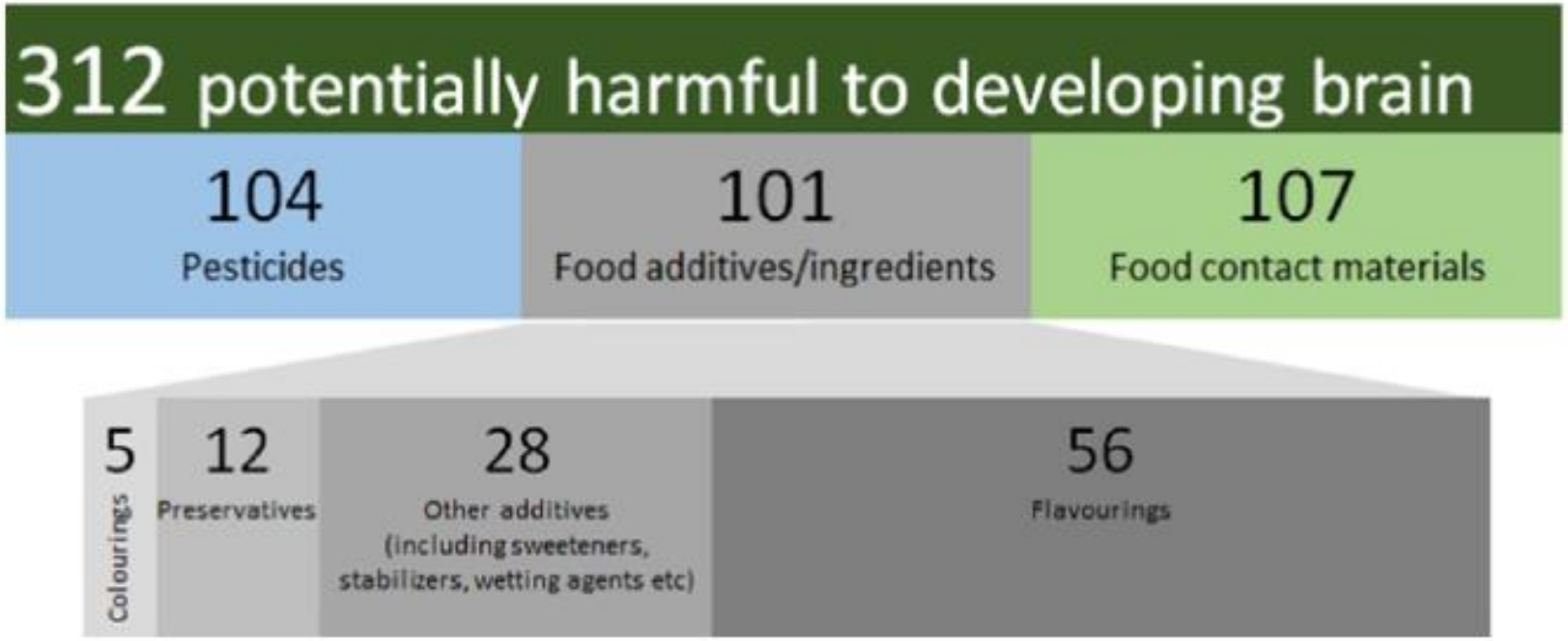
- Common, similar or related toxic effects can be shown for different chemicals that **are regulated in different silos**

- **The combined effects of chemicals across silos are not currently considered by regulation** except a few examples e.g. maximum residue limits for pesticides in food registration (Regulation EC No 1881/2006)

DNT chemicals belong to diverse chemical groups:

- Pesticides (e.g. chlorpyrifos, paraquat, DDT)
- Metals (e.g. lead, mercury, cadmium, arsenic, manganese, triethyltin)
- POPs (PCBs, PBDEs flame retardants, perfluorate-PFOS and perfluorate-PFOA)
- Endocrine disruptors (e.g. bisphenol A, perchlorate, triclosan, fluoride)
- Organic solvents (e.g. ethanol, toluene, xylene)
- Drugs (e.g. valproic acid, haloperidol, chlorpromazine, cocaine, dexamethasone)

Chemicals grouped according to food-related use are subjected to three legislations:



(Evans R., Martin O., Faust M. and Kortenkamp A., 2016; Science of the Total Environmental 543: 757-764)

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Aims of the study:

- Define whether **non-cytotoxic concentrations** of single chemicals will become neurotoxic in mixture
- Develop *in vitro* approach permitting evaluation of DNT effects induced by exposure to **mixture of chemicals** using **human neuronal/glial cultures**
- Build a battery of *in vitro* assays **anchored to common key events identified in the network of existing DNT AOPs**
- Select chemicals according to the established criteria and evaluate mixture-induced DNT effects which could contribute to **children's learning and memory impairment**
- Define ***LOAECs*** *(Lowest Observable Adverse Effect Concentrations)* value for single chemicals and in mixture.
- Determine through **mathematical modelling** whether synergistic, antagonistic or additive effects are observed in mixture

.

Grouping of chemicals based on biological or toxicological effects

"MoA and AOP data provide a strong scientific basis to group chemicals …."

Table 4: Examples of approaches for grouping chemicals

Grouping approach	***Overarching common feature***	***Example***	***Comments***
Common MoA or AOP	Toxicological or biological properties	Acetylcholine esterase inhibitors, AhR agonists, metabolism to similar bioactive parent	Chemicals acting via same pathways that converge to common molecular target (adverse outcome)

Guidance on ***Harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals*** (EFSA Scientific Committee, 2019)

EFSA have recommended that pesticides which produce **common adverse outcomes on the same target organ/system (e.g. brain) should be grouped together for the purpose of assessing cumulative risk in relation to maximum residue limit (MRL) setting (EFSA, 2013).**

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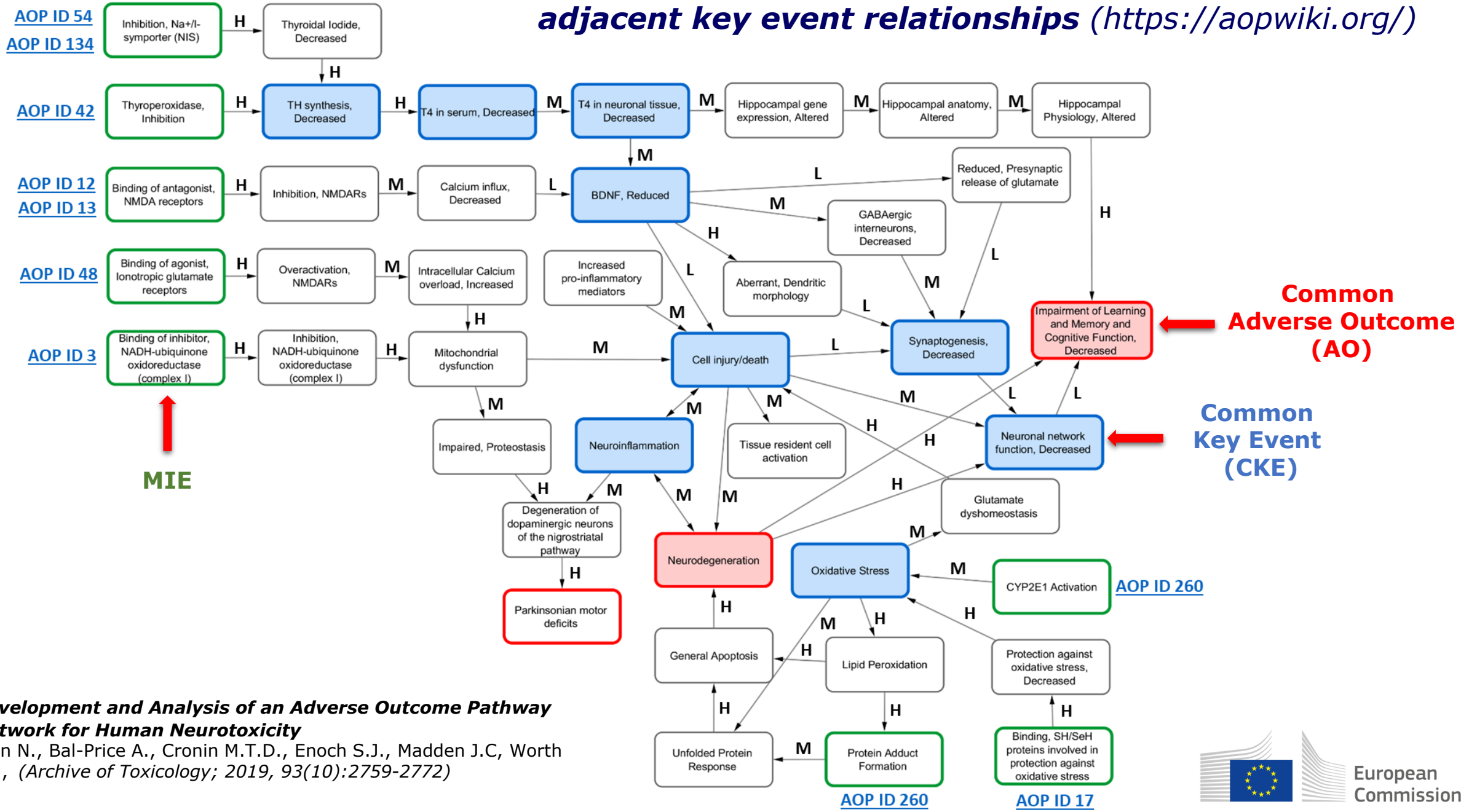
Derived network of nine AOPs for neurotoxicity containing adjacent key event relationships (https://aopwiki.org/)



Development and Analysis of an Adverse Outcome Pathway Network for Human Neurotoxicity

Spin N., Bal-Price A., Cronin M.T.D., Enoch S.J., Madden J.C, Worth A.P., *(Archive of Toxicology; 2019, 93(10):2759-2772)*

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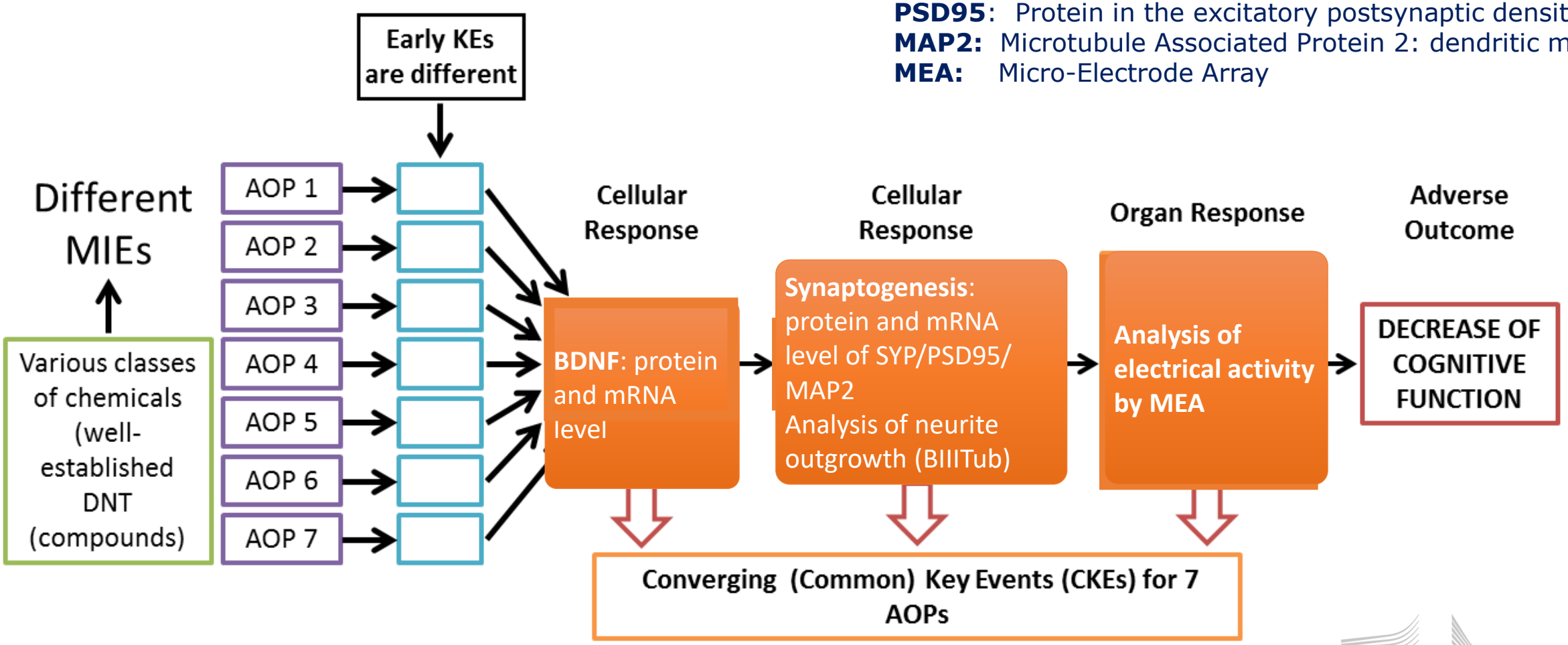
Learning and memory impairment (cognitive damage) in children: the most frequent AO of the existing AOPs relevant to DNT

BDNF: Brain Derived Neurotrophic Factor
SYP: Synaptophysin (synaptic vesicle protein)
PSD95: Protein in the excitatory postsynaptic density
MAP2: Microtubule Associated Protein 2: dendritic marker
MEA: Micro-Electrode Array



CKEs served as anchors for DNT in vitro assays

(AOP-Wiki and Bal-Price & Meek Pharmacol Ther. 2017 Nov;179:84-95)

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Criteria for chemical selection

1. **Compounds known to cause cognitive impairment (AO)**

2. **Compounds acting through identified common KEs in the AOPs**

3. **Compounds representing different classes (i.e., pesticides, industrial chemicals, heavy metals, POPs, and EDs)**

4. **Compounds found in human samples (e.g., breast milk, cord blood, urine, hair, umbilical cord plasma, brain tissues, maternal blood, or blood of children)**

5. **Compounds according to EFSA (2013) working through:**

    - **similar MoA**
    - **dissimilar MoA**

The selection of **heterogeneous** classes of chemicals

Chemicals acting through similar MoA (altered BDNF levels)		
	Chemical name	**Class**
1	**Lead(II) chloride**	**Metals**
2	**Chlorpyrifos**	**Pesticide**
3	**PBDE-47 (most abundant in human tissues)**	**POP**
4	**Ethanol**	**Organic compound Industrial chemical**
5	**Bisphenol A (BPA)**	**Organic compound (ED, estrogenic)**
Chemicals acting through dissimilar MoAs resulting in cognitive impairment		
	Chemical name	**Class**
1	**Methyl mercury chloride**	**Metals**
2	**Valproic acid**	**Antiepileptic drug**
3	**PCB-138 (most abundant in human tissues)**	**POP**
4	**Vinclozolin**	**Pesticide (ED, anti-androgenic)**
5	**TCDD**	**POP (ED, anti-estrogenic)**

Chemical concentrations tested in vitro (Phase 1) in relation to concentrations found in human samples

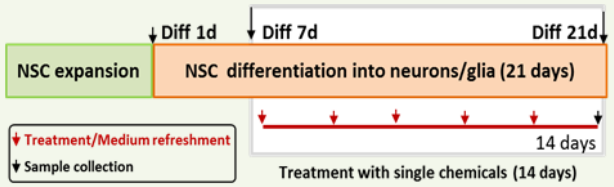
Chemical	Abbreviation	Concentrations tested *in vitro*	*Concentrations found in human samples*
Lead(II) chloride	**Lead**	200, 50, 12.50, 3.13, 0.78, 0.20 µM	**Cord blood**: range 1.09 - 11.41 µg/L →0.0039 – 0.041 µM **Children blood:** range 1.71 - 10 µg/dL →0.061 – 0.36 µM IPChem: **Blood-whole blood:** 3.76-69 , 3.42-28.8, 4.13-43.6, 6.05-23.1 µg/L (range 3.42 – 69) → 0.012 – 0.25 µM **Cord blood-whole blood** (considering for plasma, 1.025 g/mL) 2.68-36.4 ng/g → 0.00988 – 0.13 µM
Chlorpyrifos	**CPF**	500, 125, 31.25, 7.81, 1.95, 0.49 µM	**Cord plasma:** 4.65 ng/mL → 0.013 µM **Cord blood:** range 2.5 – 6.17 pg/g plasma (considering for plasma, 1.025 g/mL) → $7.3x10^{-6}$ - $1.8x10^{-5}$ µM
Bisphenol A	**BPA**	400, 100, 25, 6.25, 1.56, 0.39, 0.10 µM	**Children serum**: range 0.85 - 22.5 ng/mL → 0.0037 - 0.098 µM IPChem: **Blood – plasma**: n.d.- 3.5 ng/g (considering for plasma, 1.025 g/mL) → n.d. - 0.016 µM Cord blood-whole blood: n.d.-1.9 ng/g (considering for plasma, 1.025 g/mL) → n.d. - 0.0085 µM
Methyl-mercury(II) chloride	**Methyl-Hg**	10, 2.50, 0.63, 0.16, 0.04, 0.01, 0.0024, 0.0006 µM	**Cord blood**: range 0.70 - 35 µg/L → 0.0028 - 0.14 µM **Children blood**: range 1.46 - 6.81 µg/L → 0.0058 – 0.027 µM IPChem: **Blood-whole blood**: 0.11-10.2, 0.002-4.17, 0.19-7.93, 0.13-5.95 µg/L (range 0.002 – 10.2) → $8x10^{-6}$ - 0.041 µM **Cord blood-whole blood**: 0.16 - 14.1 ng/g (considering for plasma, 1.025 g/mL) →0.00065 – 0.058 µM, **Blood –plasma**: n.d. - 4.2 µg/L → n.d.- 0.017 µM
Valproic acid	**VA**	10.000, 2500, 625, 156, 39, 10 µM	**Cord blood:** range 3.87 - 75 µg/ml → 26.8 - 520 µM
PCB138	**PCB138**	100, 25, 6.25, 1.56, 0.39, 0.10, 0.02 µM	**Cord plasma**: range 0.14 – 0.18 ng/mL → $3.87x10^{-4}$ - $5x10^{-4}$ µM IPChem: **cord plasma**: 270 - 460 ng/L → 0.00075 – 0.0013 µM

The experimental design

Phase I

Evaluation of cytotoxicity for single chemical treatments

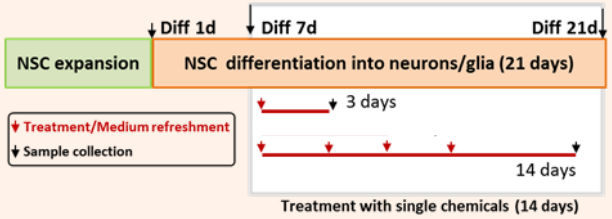
To define non-cytotoxic concentrations, low level toxicity ($IC_{20}/100$, IC_5), toxic (IC_{20}), with solvent control (0.1% DMSO)



(CellTiter Blue)

Phase II

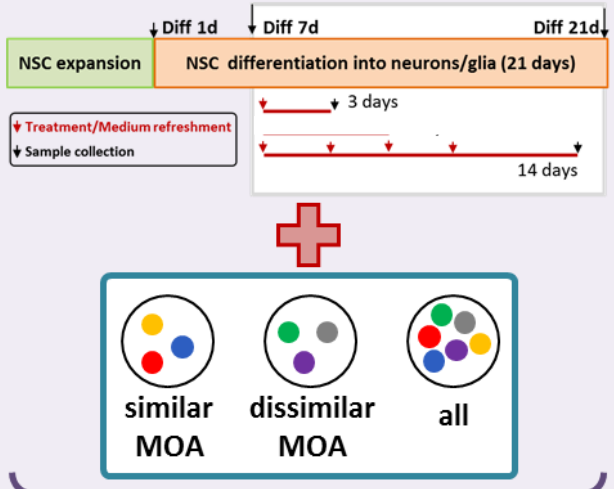
Repeated dose treatments with single compounds and analysis of DNT specific endpoints: synaptogenesis, neurites and BDNF expression



Goal: *Define LOAECs (Lowest Observable Adverse Effect Concentrations) based on statistical significance (one-way Anova plus Dunnet post-hoc test)*

Phase III

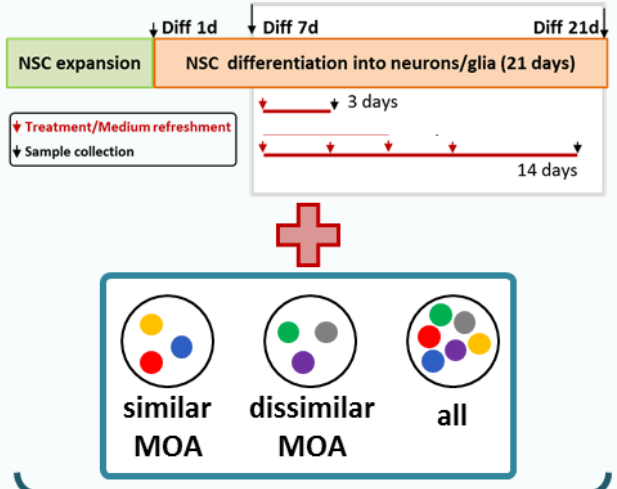
Repeated dose treatments with mixed compounds and analysis of DNT specific endpoints: synaptogenesis, neurites and BDNF expression



Additive
Synergistic
Antagonistic effects

Phase IV

Comparative analysis: mixed compounds at relevant exposure concentrations



Additive
Synergistic
Antagonistic effects

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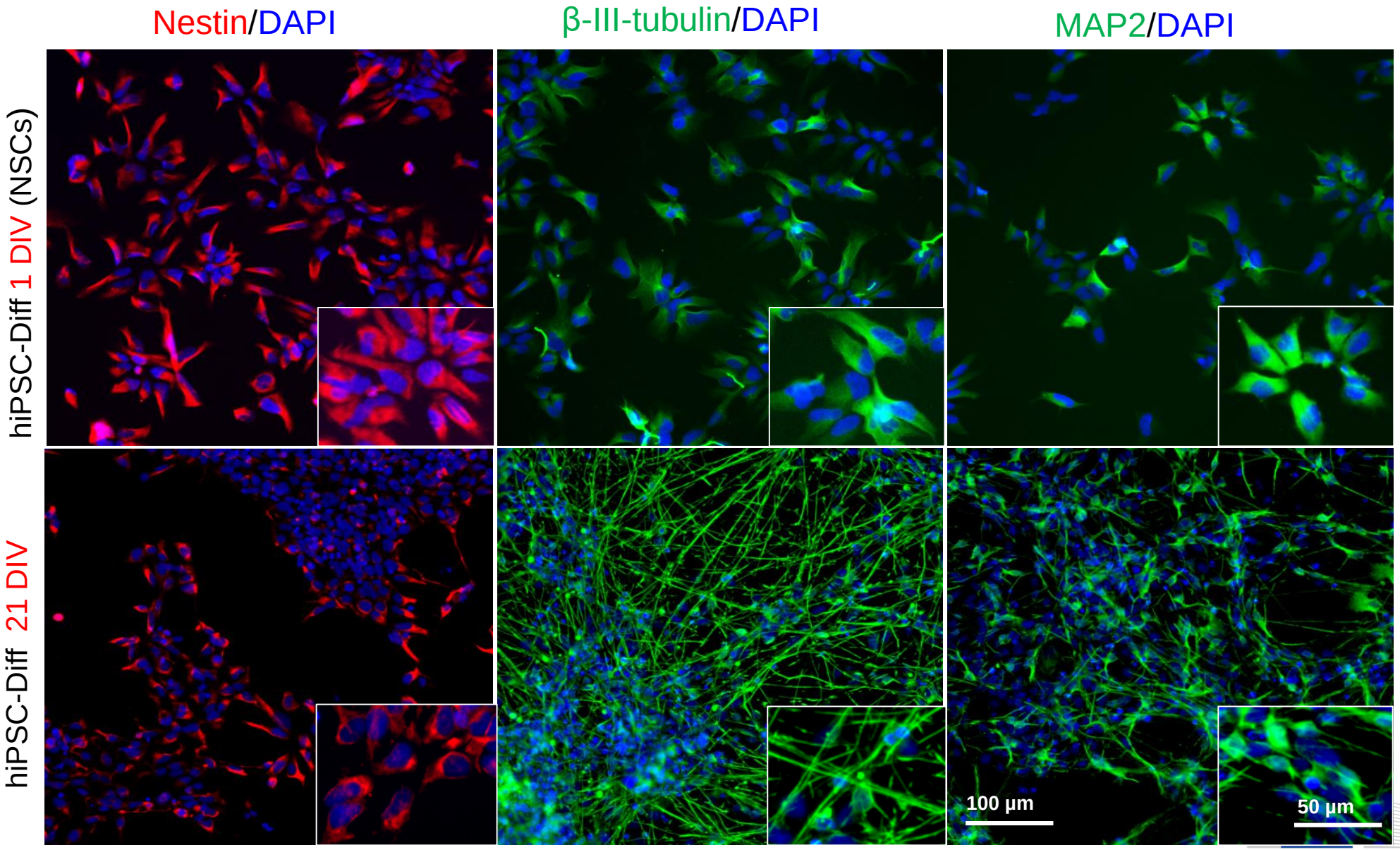
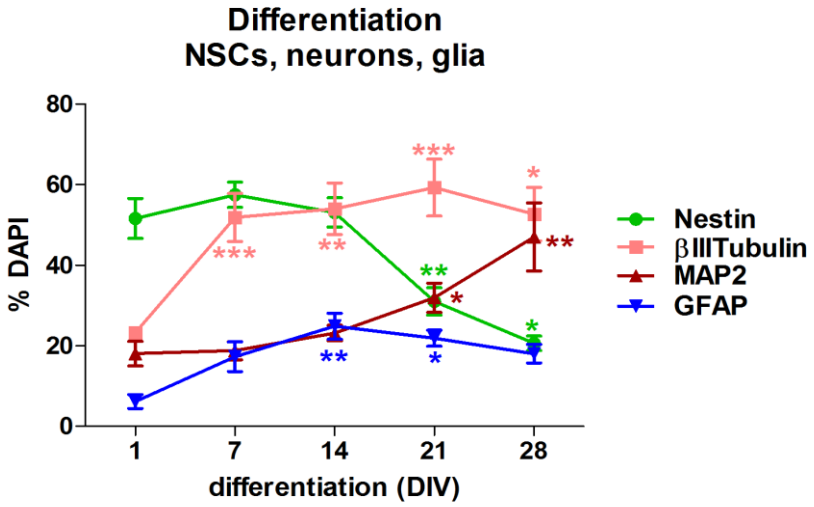
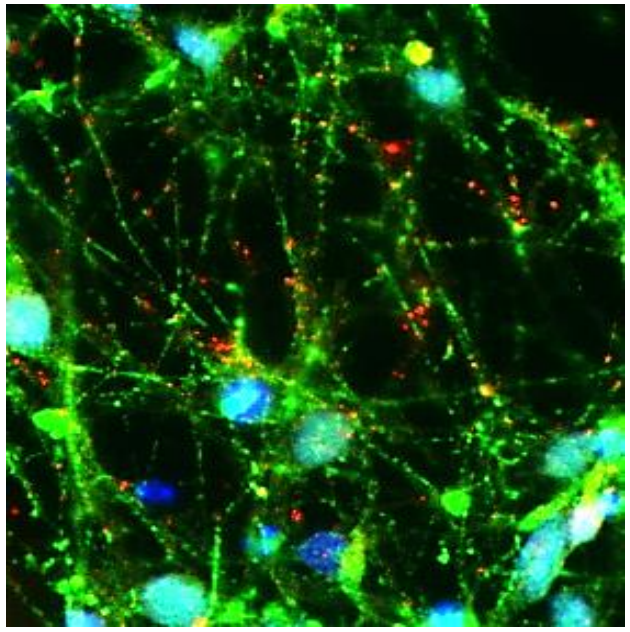
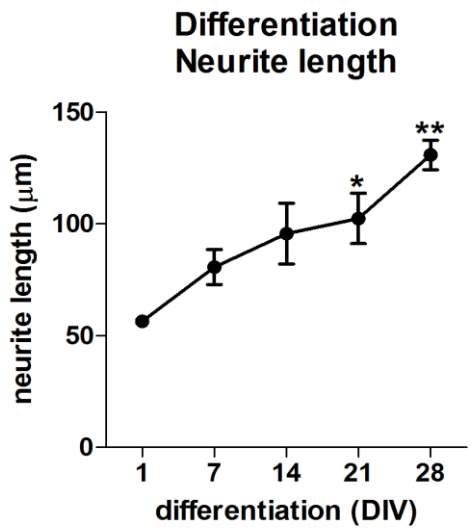
Differentiation of mixed neuronal/glial cells derived from human NSCs (iPSCs)

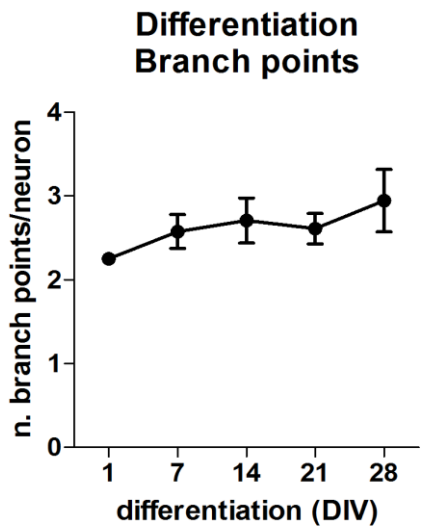


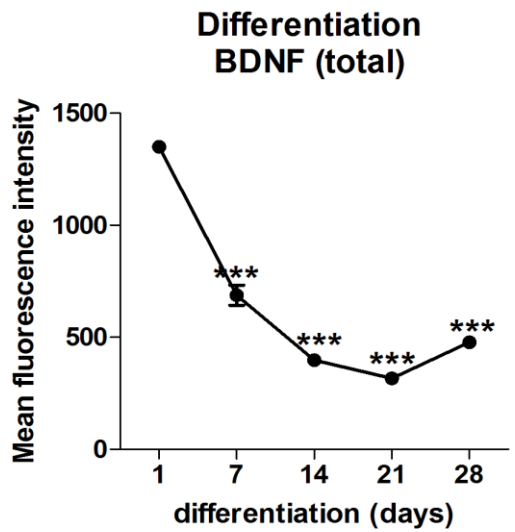
Characterization of the control, mixed, neuronal/glial culture

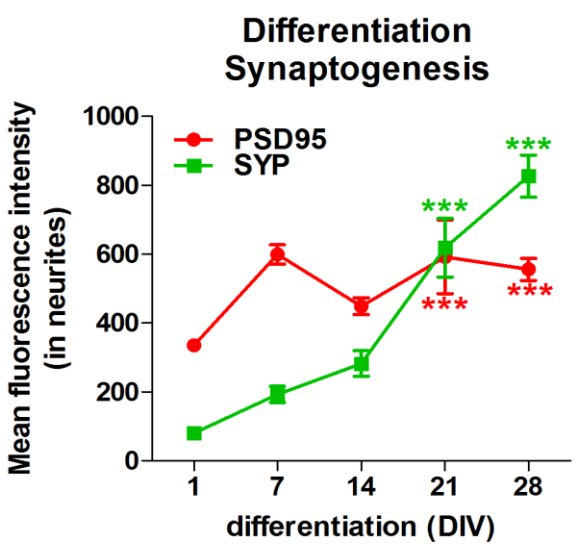


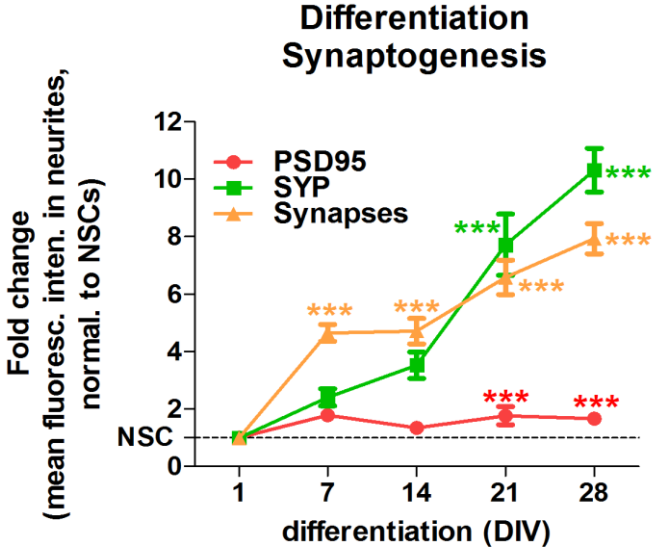












Data are shown as mean ± S.E.M. of three biological replicates (p < 0.05, ** p < 0.01, *** p < 0.001).*

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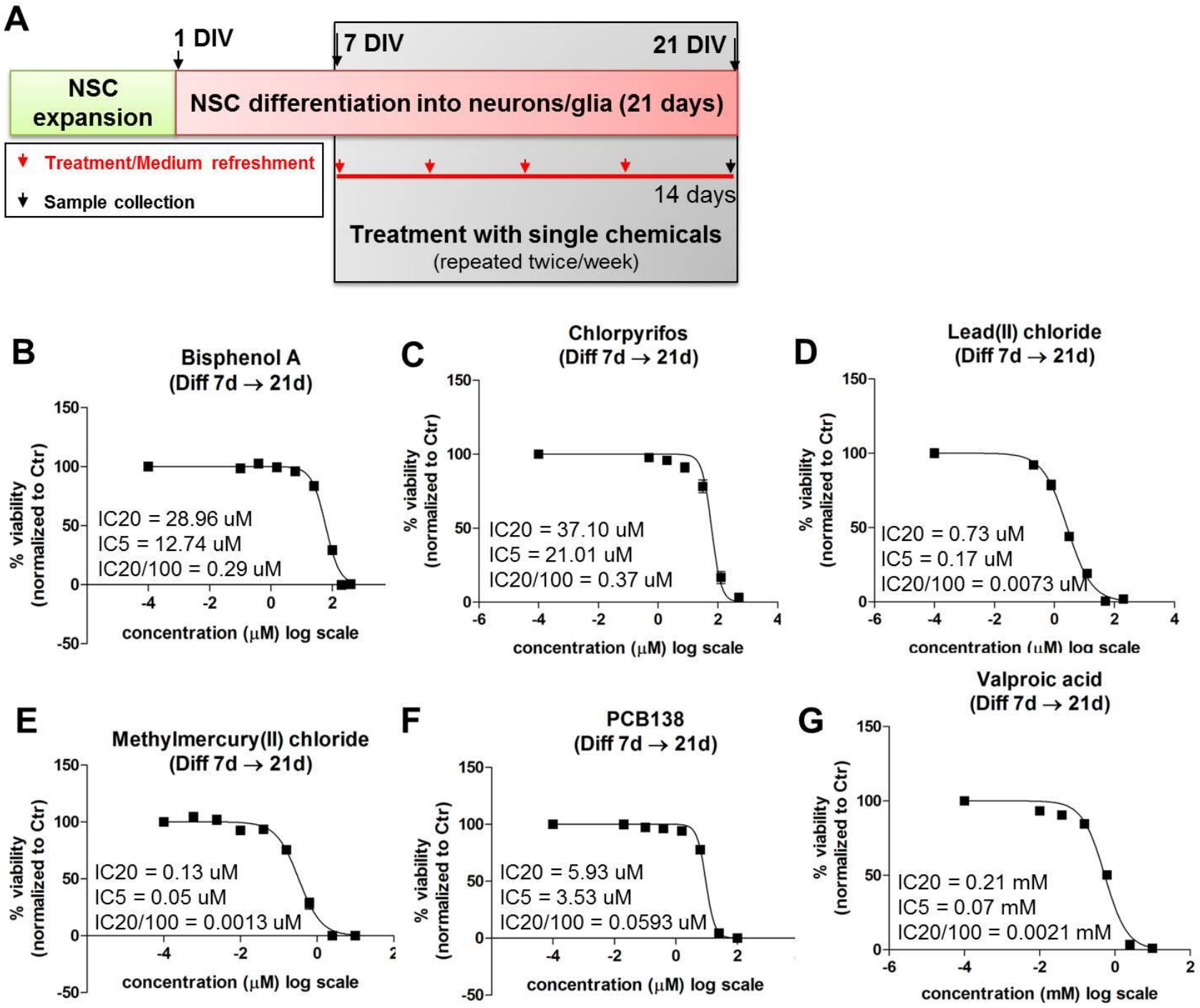
Phase 1

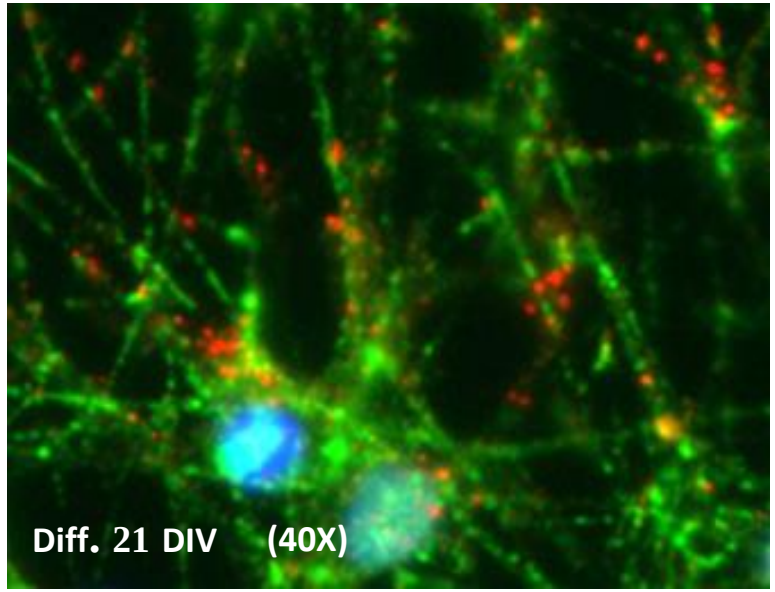
Cell viability upon treatments with single chemicals

Non-cytotoxic concs, $IC_{20}/100$, IC_5 and IC_{20} selected for the Phase II

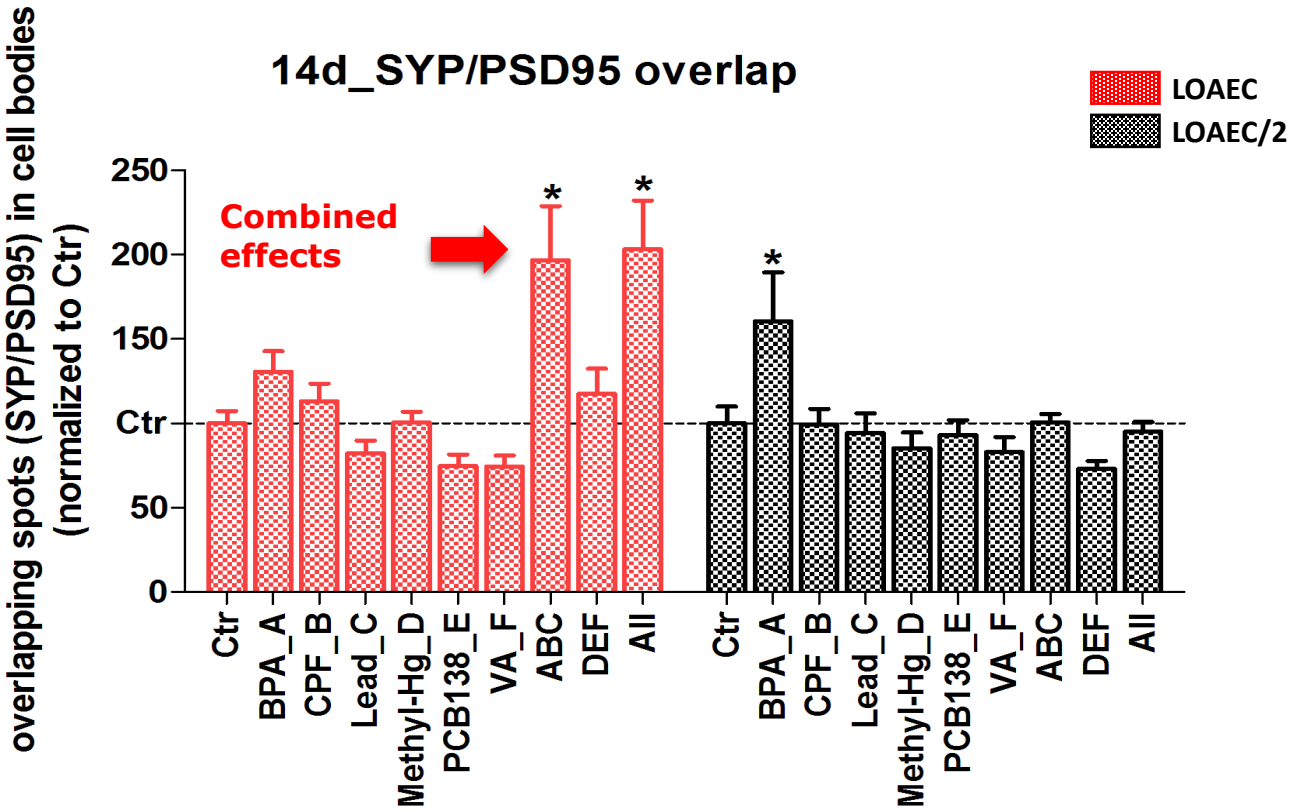


Phase II & III (some representative results)

Synaptogenesis



PSD95 SYP DAPI

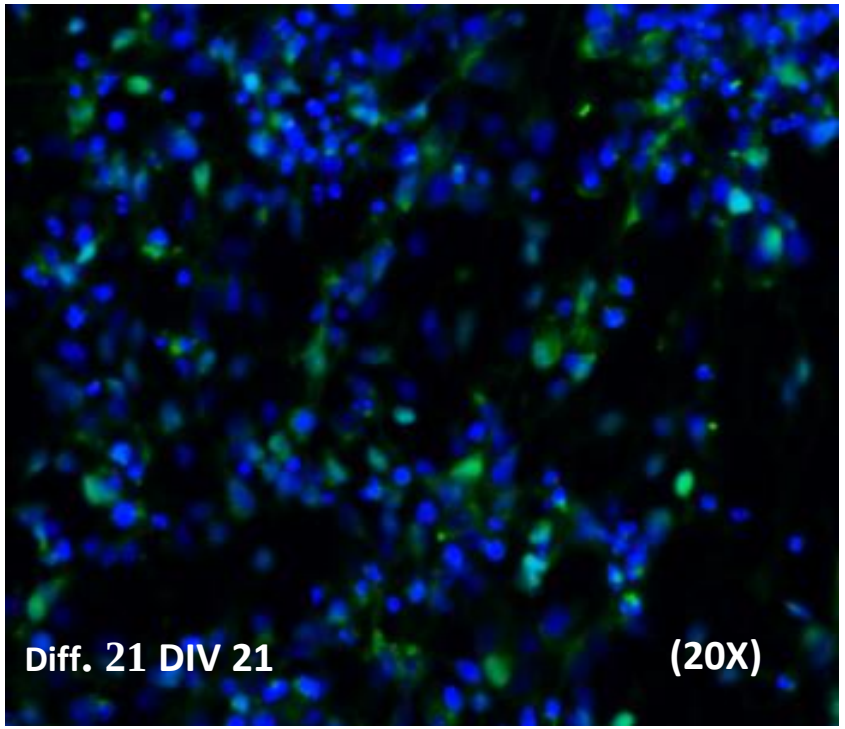


BPA	Chlorpyrifos	Lead (II)-Cl	Methyl -Hg	PCB138	Valproic Ac.	(μM)
12.0	21.0	0.007	0.0500	0.06	2.1	LOAEC
6.0	10.5	0.0035	0.025	0.03	1.05	LOAEC/2

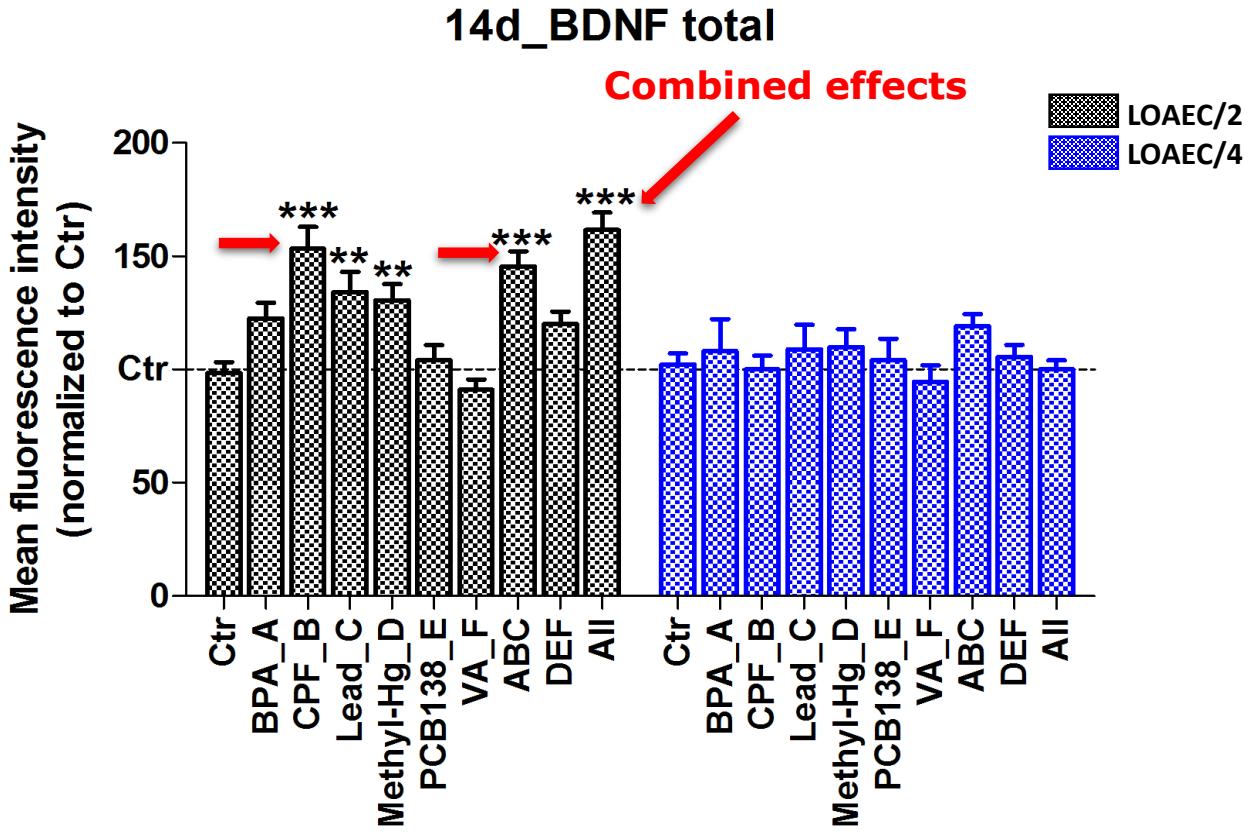
Main (stat. signif.) effects:

- "ABC" and "All" mix increased PSD95/SYP co-localization (at LOAEC)

BDNF protein levels



BDNF/DAPI



BPA	Chlorpyrifos	Lead(II)-Cl	Methyl-Hg	PCB138	Valproic Ac	(μM)
6.4	18.5	0.7	0.06	1.8	105	LOAEC/2
3.2	9.25	0.35	0.03	0.9	52.5	LOAEC/4

Main (stat signif) effects:

- CPF alone is the strongest inducer of BDNF expression, followed by Lead (at LOAEC/2)
- CPF drives toxicity of mixture (confirmed by exposure to ABC and All without CPF)

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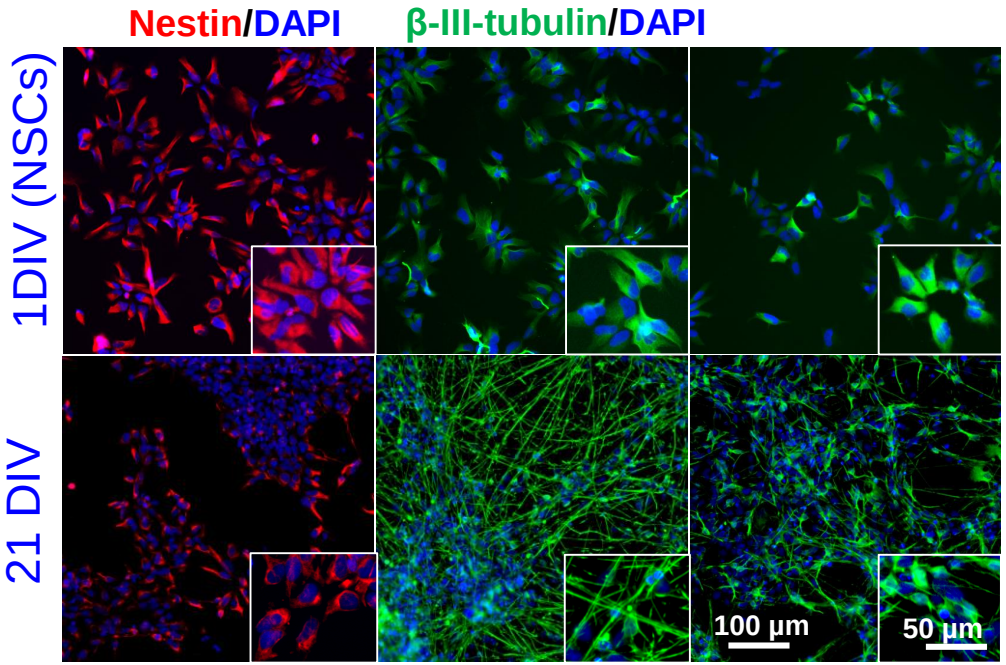
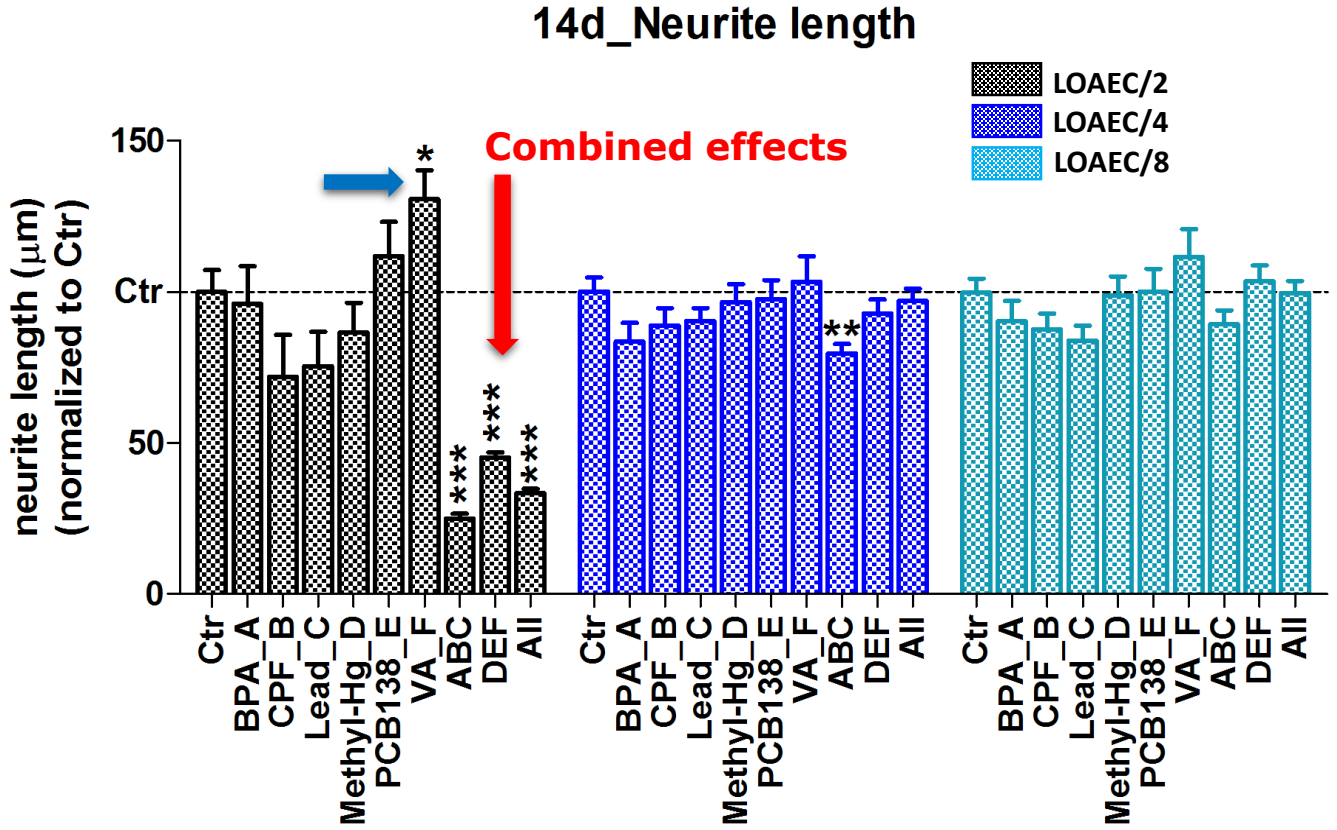
Neurite outgrowth





BPA	Chlorpyrifos	Lead(II)-Cl	Methyl-Hg	CB138	Valproic Ac	(µM)
6.4	37.0	0.6	0.06	6.0	210	LOAEC/2
3.2	18.0	0.3	0.03	3.0	105	LOAEC/4
1.6	9.0	0.15	0.015	1.5	52.5	LOAEC/8

Main (stat. signif.) effects:

- ABC, DEF and ALL mix downregulates neurite length at LOAEC/2 and ABC only at LOAEC/4
- CPF drives the toxicity of the mix, followed by Lead
- VA increases neurite outgrowth features (LOAEC/2)
  (→ **VA may have antagonistic effects in "DEF" and "All" mix**)

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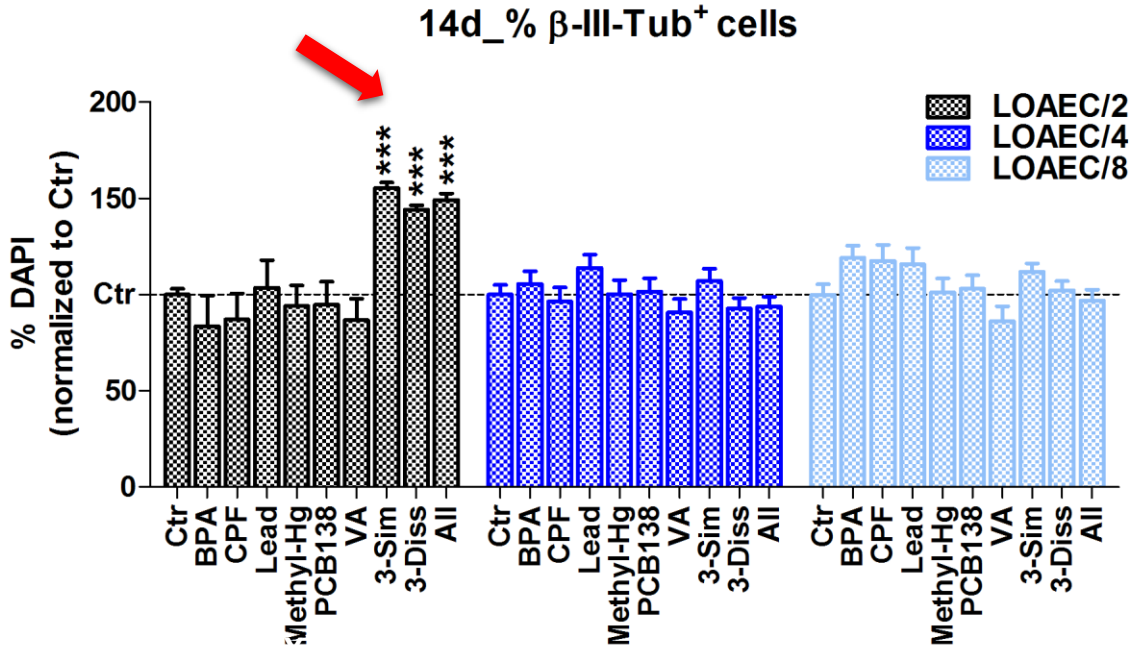
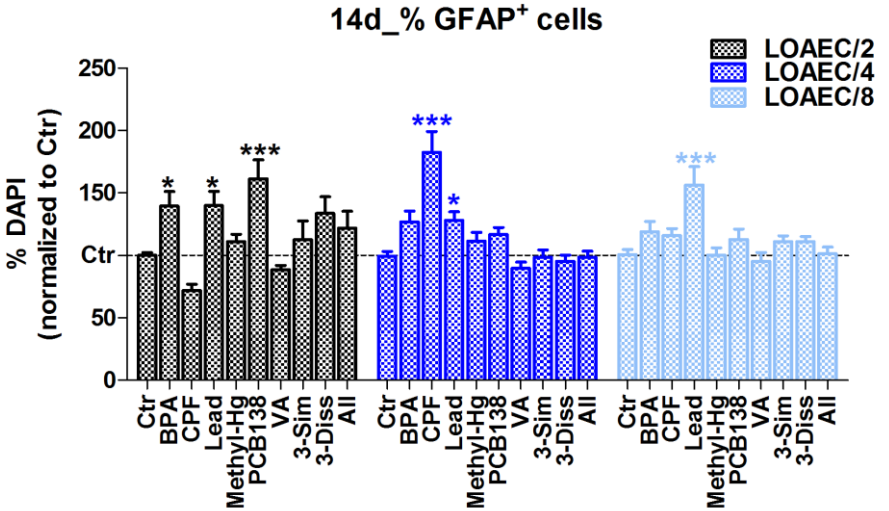
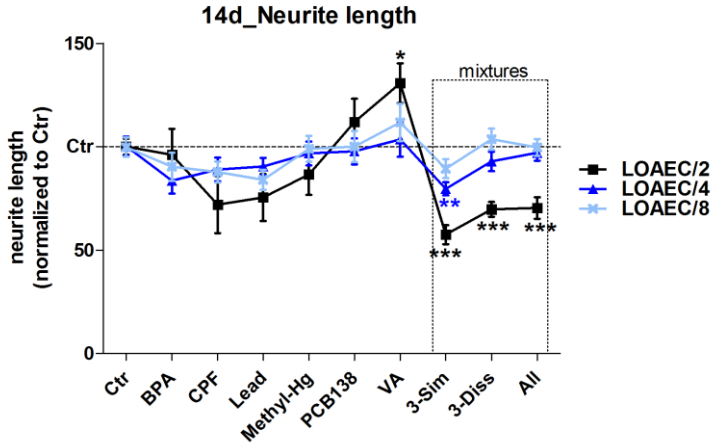
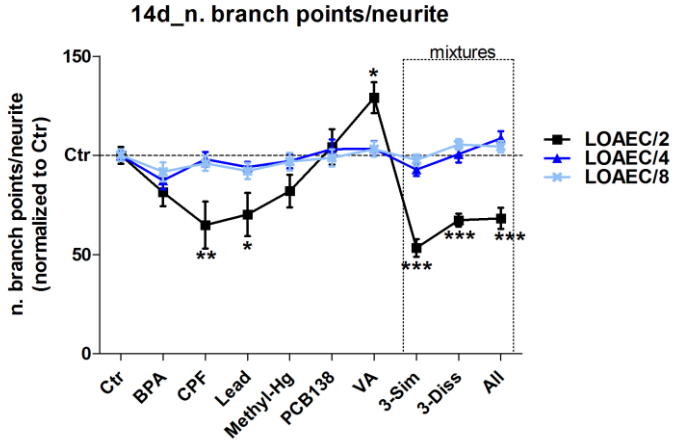
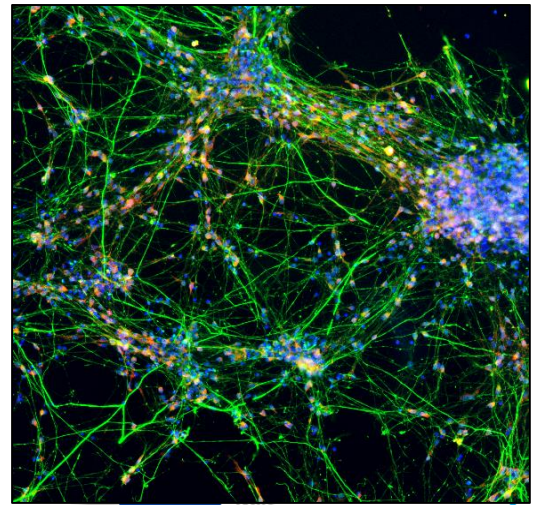
Significant augmentation of neuronal cells number (by ~55%) after exposure to mixtures











GFAP/NF200/DAPI (21 DIV)

Alternations observed in the children's brain with autism spectrum disorder (ASD):

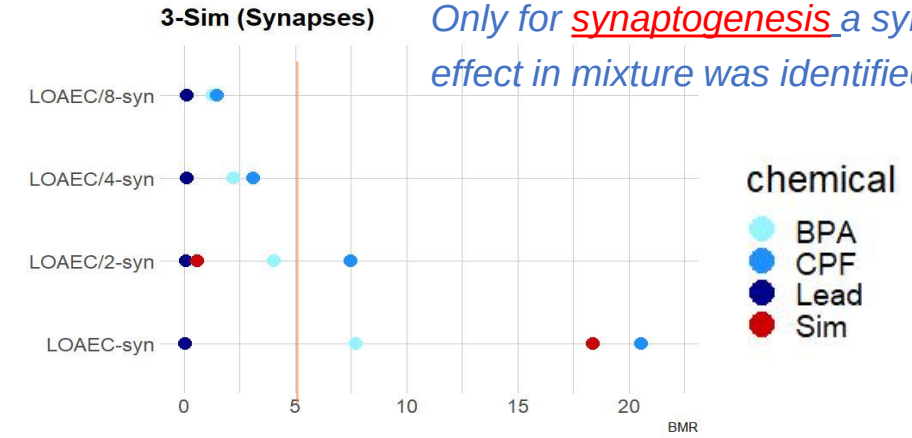
- **An increase of neuronal cell number** in the prefrontal cortex (approx. 67%) (Courchesne et al., 2010) (observed in vitro)

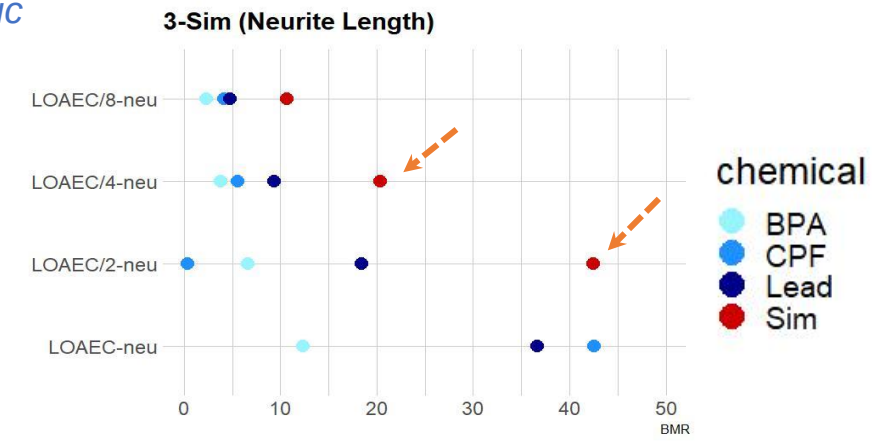
- **Impaired neurite morphology**: shorter and less branched neurites (Nguyen et al., 2018; Nagy et al., 2017) (observed in vitro)

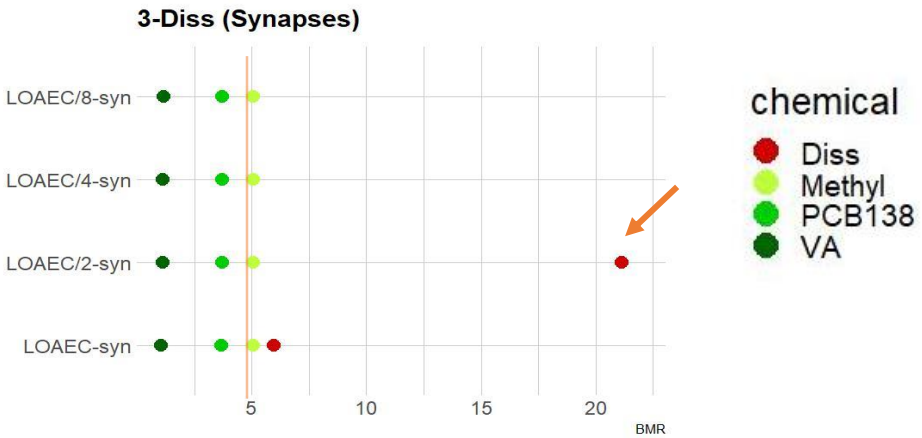
  Approx. 80% of the genes linked to ASD play an important role in early neurodevelopmental functions, neurite outgrowth and synapse formation (Casanova et al., 2014)

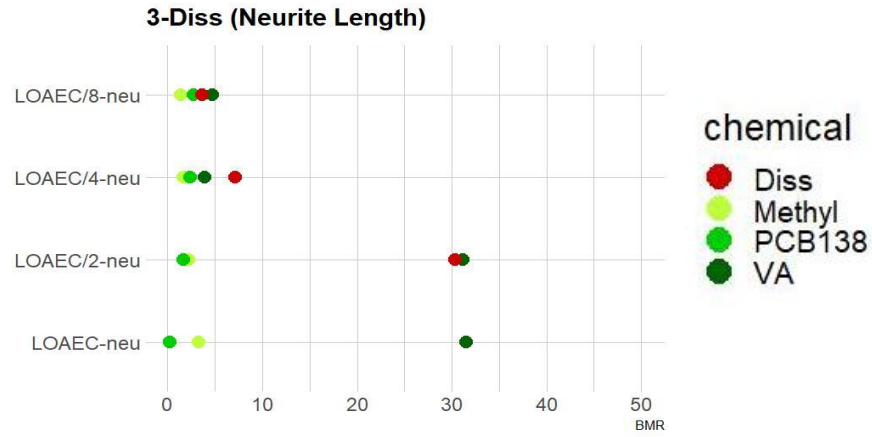
- **Elevated BDNF levels** both in peripheral blood (Bryn et al., 2015) and in the frontal cortex (Maussion et al., 2019) as confirmed by recent meta-analyses (Saghazadeh et al., 2017; Armeanu et al., 2017) (observed in vitro)

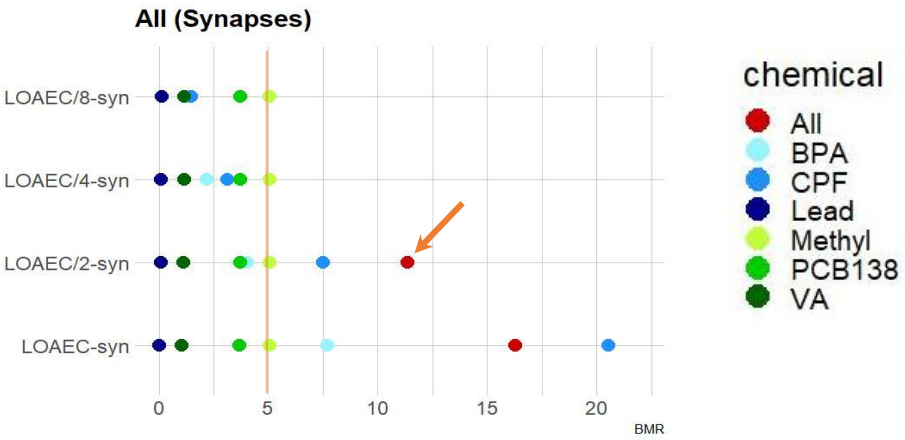
Only for synaptogenesis a synergistic effect in mixture was identified.

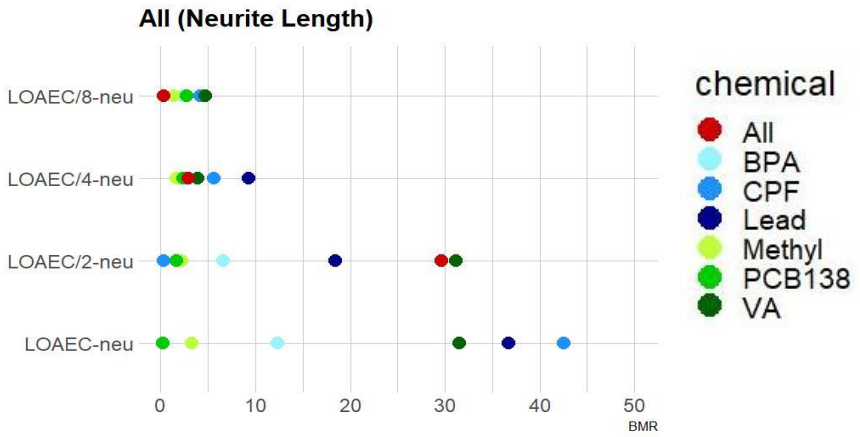












Bench Mark Dose Modelling (14d exposure)

Lowest Akaike model;
BMR value
BMD5 &
Threshold :
Toxic Unit (TU) <1

"Adverse outcome pathway-driven assessment of developmental neurotoxicity induced by chemical mixtures using human stem cell-derived neuronal/glial cultures"
Pistollato et al., ***Environmental Health****, 2020 (in press)*

European Commission

Conclusions:

- Low concentrations (i.e., below LOAECs) of single chemicals (non-neurotoxic) become neurotoxic in mixture, especially for the chemicals working through similar MoA
- Synaptogenesis seems to be the most sensitive DNT endpoint: synergistic effect observed in mixture after 14 day exposure to similar MoA chemicals
- Our approach allowed to identify LOAECs for single chemicals and in mixture
- Human neurons exposed to mixture of chemicals at low concentrations reproduces some autism-like phenotypic feature (increased number of neurons, decreased neurite outgrowth, increased BDNF levels, etc.)
- Common Key Events identified in DNT AOPs network guided selection of the in vitro assays, permitting mechanistic understanding of toxicity
- The obtained data will be used for refining the existing AOPs.

Conclusion: AOPs networking is a suitable framework for evaluation DNT effects induced by mixture of chemicals

Chemicals relevant to human exposure

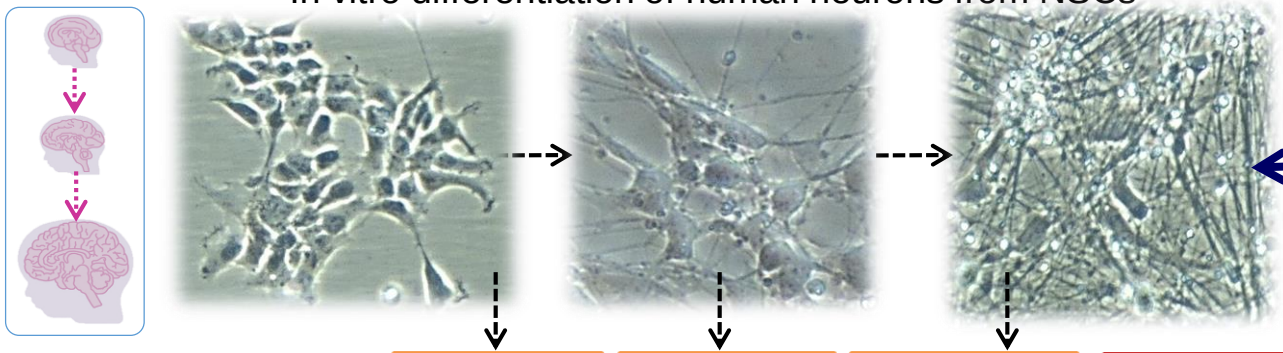
CPF (pesticide)
BPA (organic compound)
VA (drug)
Met-Hg, Pb (heavy metals)
PCB138 (POP)
3-Similar MoA
3-Dissimilar MoA
ALL

Non-toxic

EXPOSURE (3 or 14 days)
single chemicals | mixtures

Toxic

In vitro differentiation of human neurons from NSCs



In vitro model relevant to human brain development (differentiation of human NSCs represents key neurodevelopmental processes)

Mechanistic understanding: in vitro assays anchored to common KEs

Altered BDNF levels
Altered neurite outgrowth
Altered synaptogenesis
Adverse outcome

AOP network: multiple MIEs relevant to mixture exposure

AOPs network

MIE → KEs
MIE → KEs
MIE → KEs
etc. → etc.

Common Key Event → Common Key Event → Common Key Event → Cognitive deficits in children

AO of regulatory relevance

Human *in vitro* model and assays suitable to identify chemicals with potential to cause learning and memory impairment in children.

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Thank you for your attention!

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