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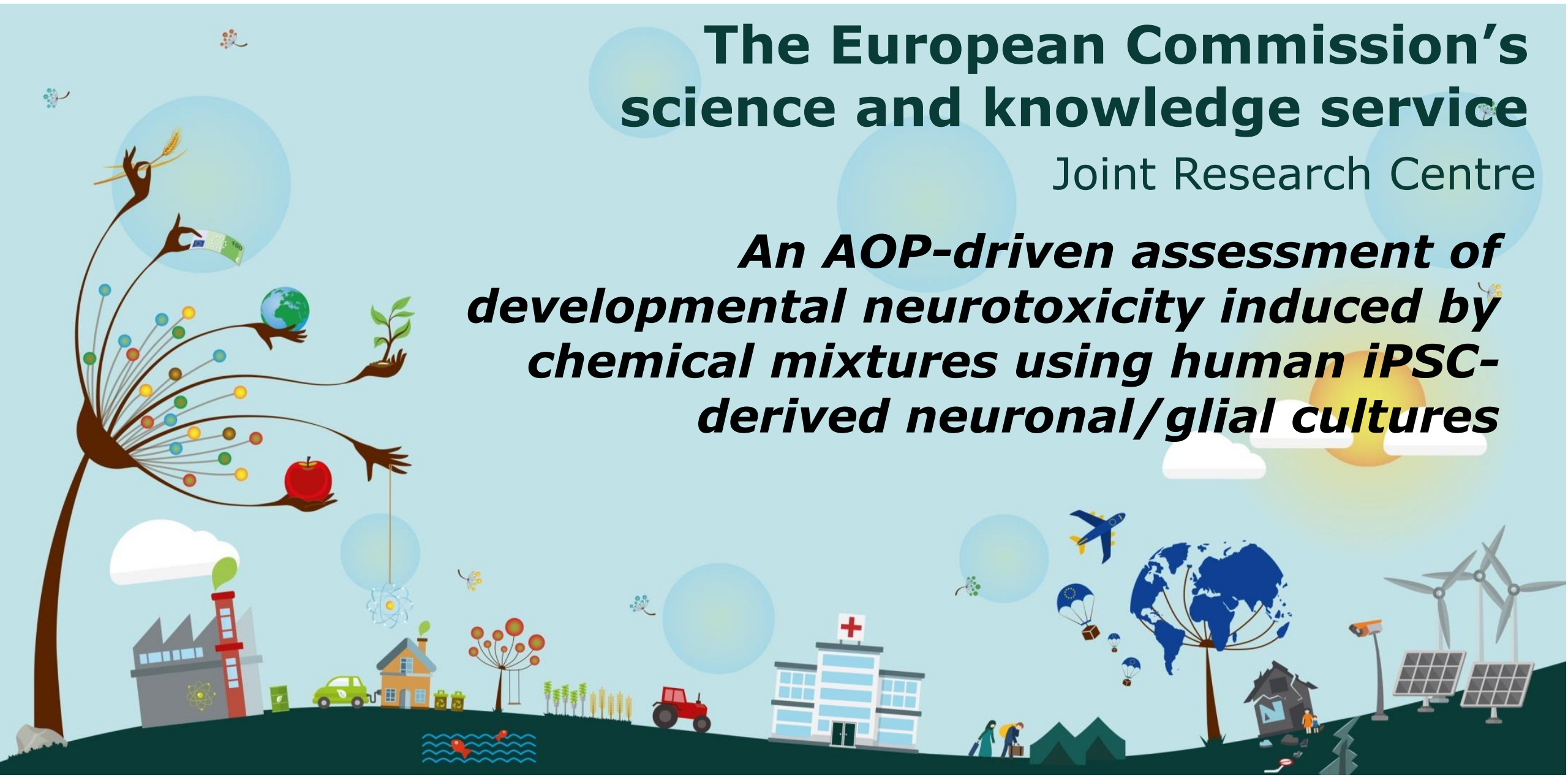

An AOP-driven assessment of developmental neurotoxicity induced by chemical mixtures using human iPSC-derived neuronal/glial cultures

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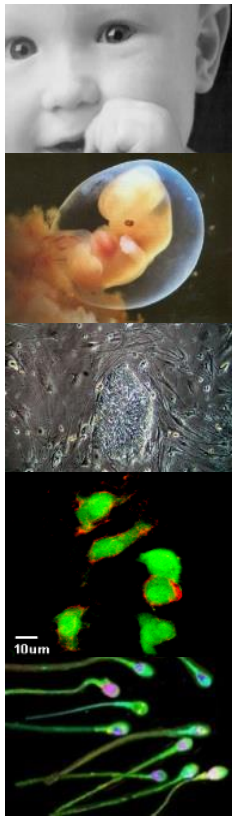
Developmental NeuroToxicity (DNT): evidence for increasing incidence of neurodevelopmental disorders

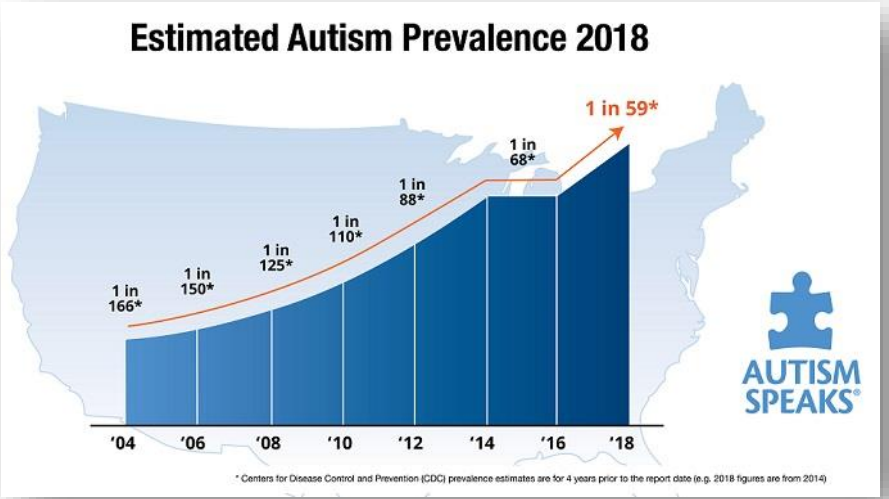


- **1 every 6 children** has a developmental disability that affect the CNS *(Atladottir et al., 2015)*

- **Decreased learning and memory capacity, autism spectrum disorders (ASD), attention-deficit/hyperactivity disorder (ADHD), dyslexia,** etc.

- Overall estimates that **10-15%** children are affected *(Grandjean & Landrigan, Lancet, 2014)*

*Genetic factors account for no more than **30–40%** (NRC, 2000)*



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
  - 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**

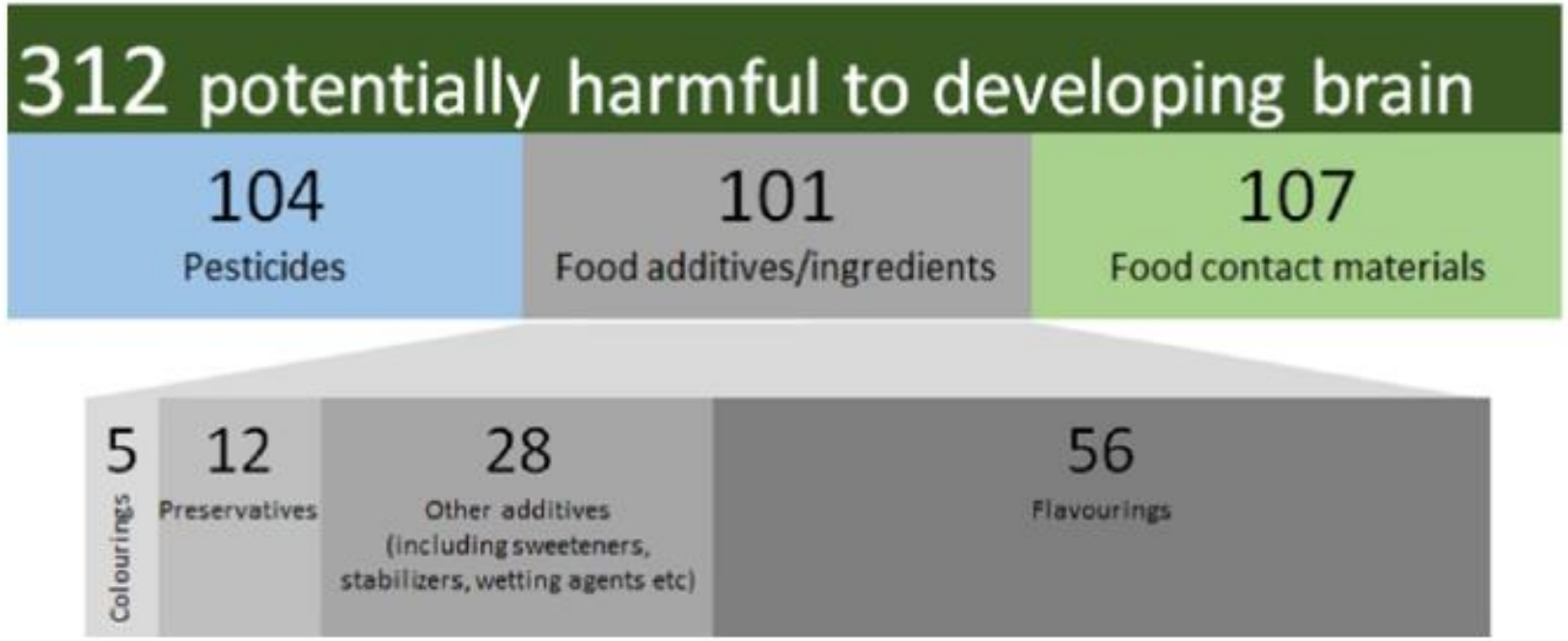
- Mixture effects are defined as either an effect greater than the most potent single chemical in the mixture, or an effect that is additive or synergistic

- 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:

- Determine whether non-neurotoxic concentrations of single chemicals will produce DNT effects in mixtures

- Define *LOAECs (Lowest Observable Adverse Effect Concentration)* value for single chemicals and in mixture using DNT specific endpoints

- Build a battery of *in vitro* assays anchored to common key events identified in the DNT AOPs network using human neuronal/glial culture to identify chemicals associated with impairment of learning and memory in children

Grouping of chemicals based on biological or toxicological effects

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

Table 3: Examples of approaches for grouping chemicals

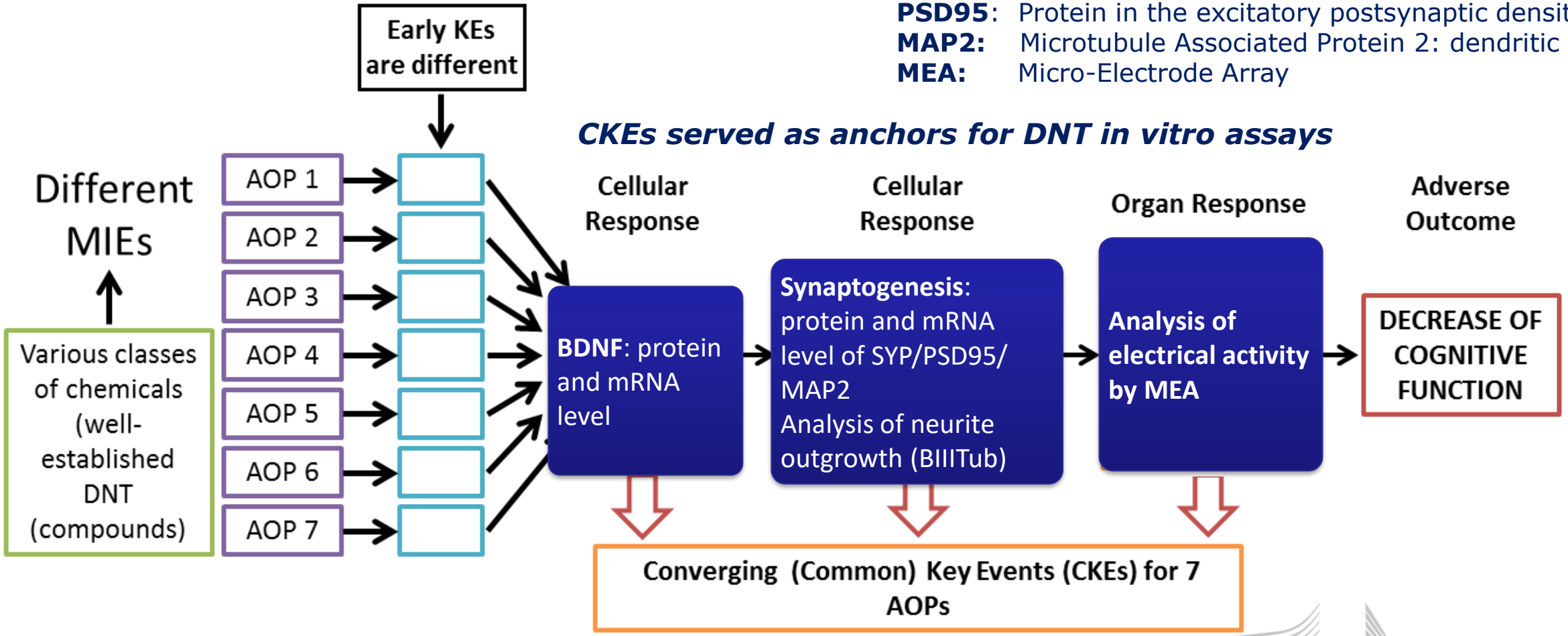
Grouping approach	***Overarching common feature***	***Example***	***Comments***
Common MoA or AOP	Toxicological or biological properties	Acetylcholine esterase inhibitors, AhR agonists, metabolism to similar bioactive metabolite(s)	Chemicals acting via same pathways that converge to common molecular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

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 (pre-synaptic vesicle protein)
PSD95: Protein in the excitatory postsynaptic density
MAP2: Microtubule Associated Protein 2: dendritic marker
MEA: Micro-Electrode Array



Bal-Price A., Pistollato F., Munn Sh., Bopp S., Worth A. Strategies to improve the regulatory assessment of Developmental Neurotoxicity (DNT) using *in vitro* methods. *2018, TAAP, 354: 7-18, TAAP*

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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 (inhibits GABA transaminase)**
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

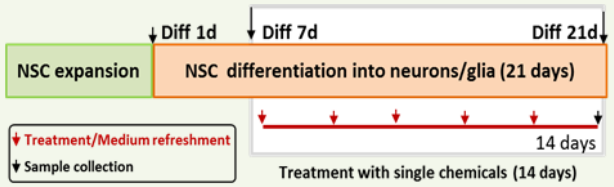
Chemical	Abbreviation	Concentrations tested *in vitro*	*Concentrations found in human samples*
Lead(II) chloride	**Lead**	200, 50, 12.50, 3.13, 0.78, 0.20 µM	**Cord blood**: range 1.09 - 11.41 µg/L →0.0039 – 0.041 µM **Children blood:** range 1.71 - 10 µg/dL →0.061 – 0.36 µM IPChem: **Blood-whole blood:** 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

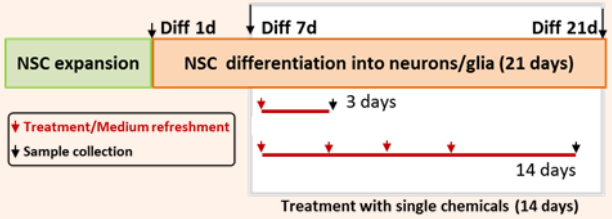
To define non-cytotoxic concentrations, low level toxicity ($IC_{20}/100$, IC_5), toxic (IC_{20}), with solvent control (0.1% DMSO)



(CellTiter Blue)

Phase II

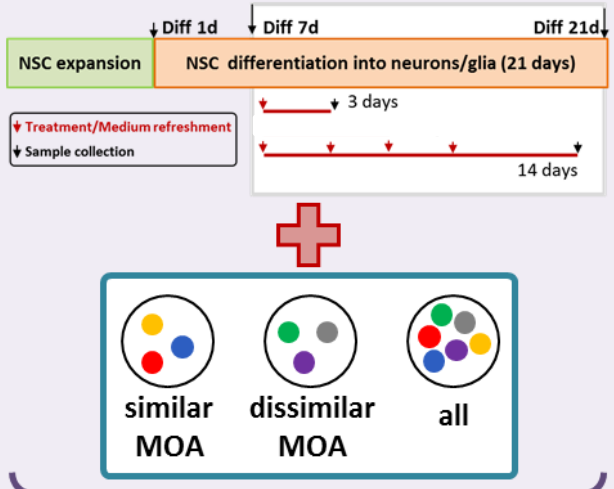
Repeated dose treatments with single compounds and analysis of DNT specific endpoints: synaptogenesis, neurites and BDNF expression



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

Phase III

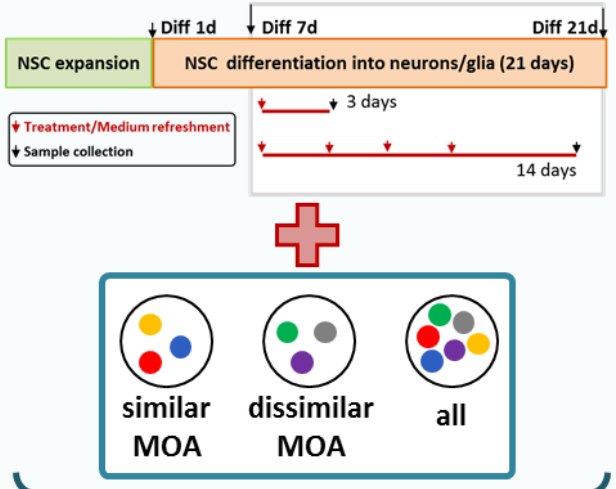
Repeated dose treatments with mixed compounds and analysis of DNT specific endpoints: synaptogenesis, neurites and BDNF expression



Additive
Synergistic
Antagonistic effects

Phase IV

Comparative analysis: mixed compounds at relevant exposure concentrations



Additive
Synergistic
Antagonistic effects

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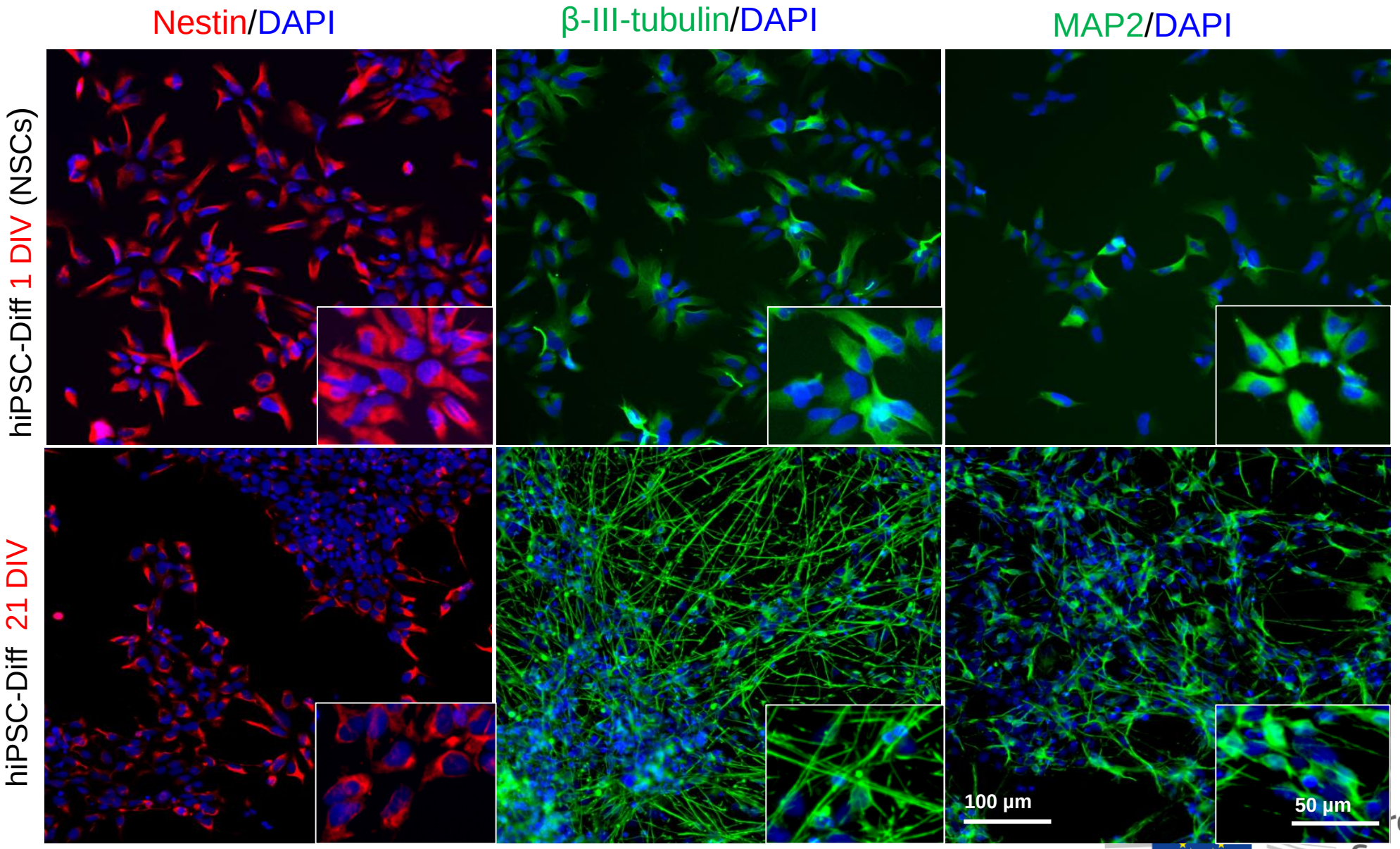
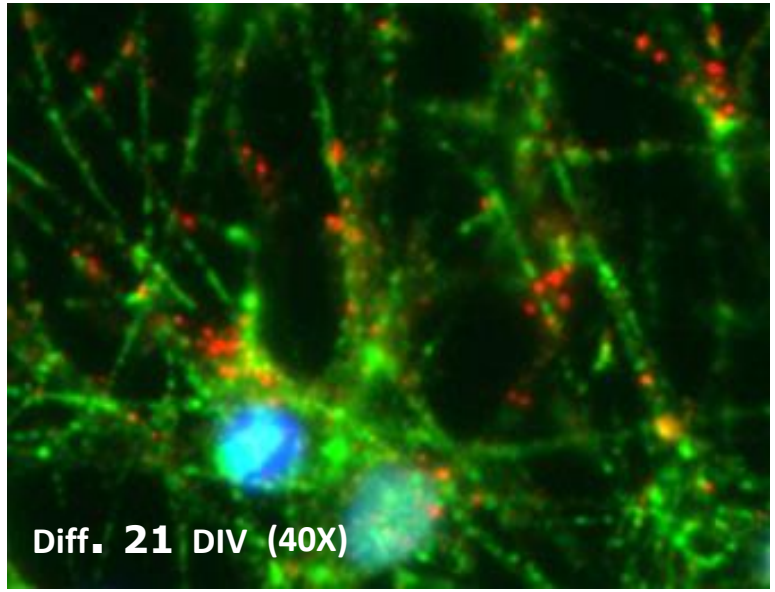
Differentiation of mixed neuronal/glial cells derived from human NSCs (iPSCs)



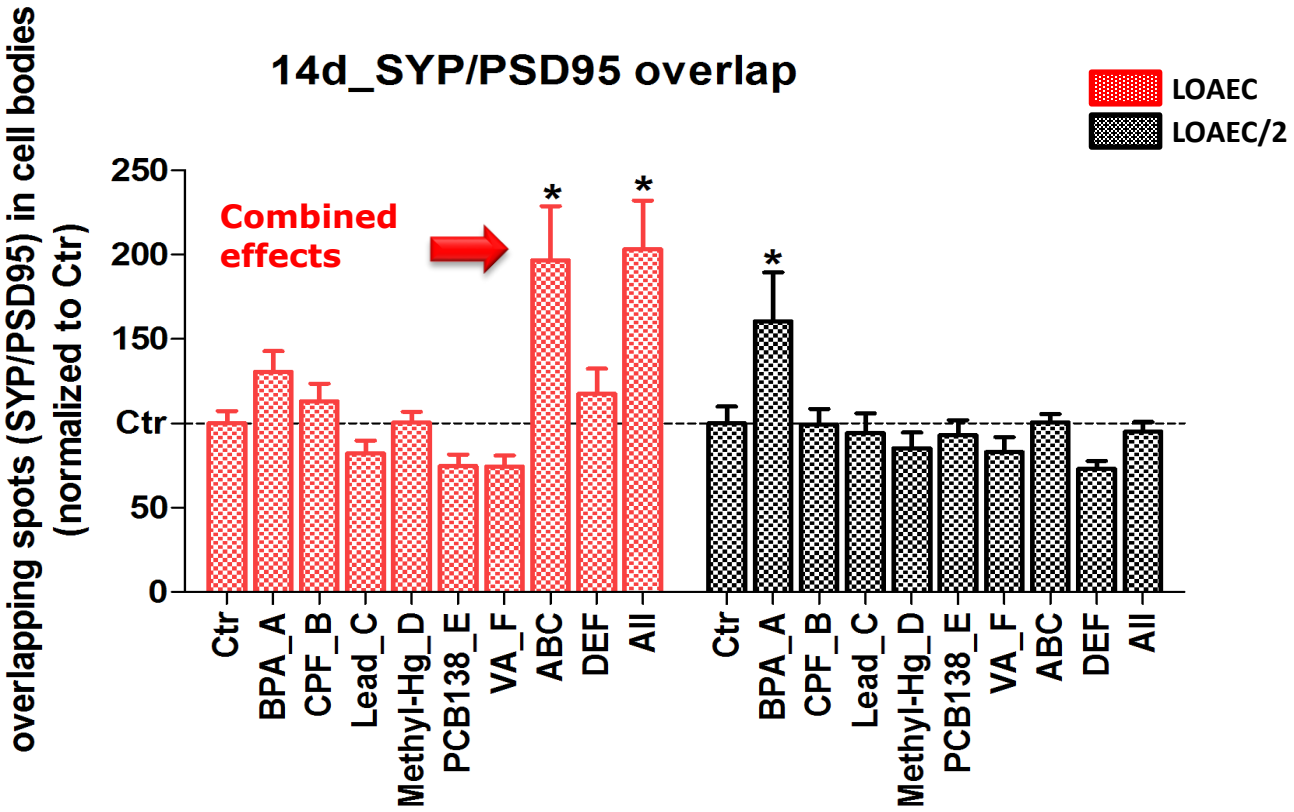
Phase II & III (some representative results)

Synaptogenesis

(14 days exposure)



PSD95 SYP DAPI

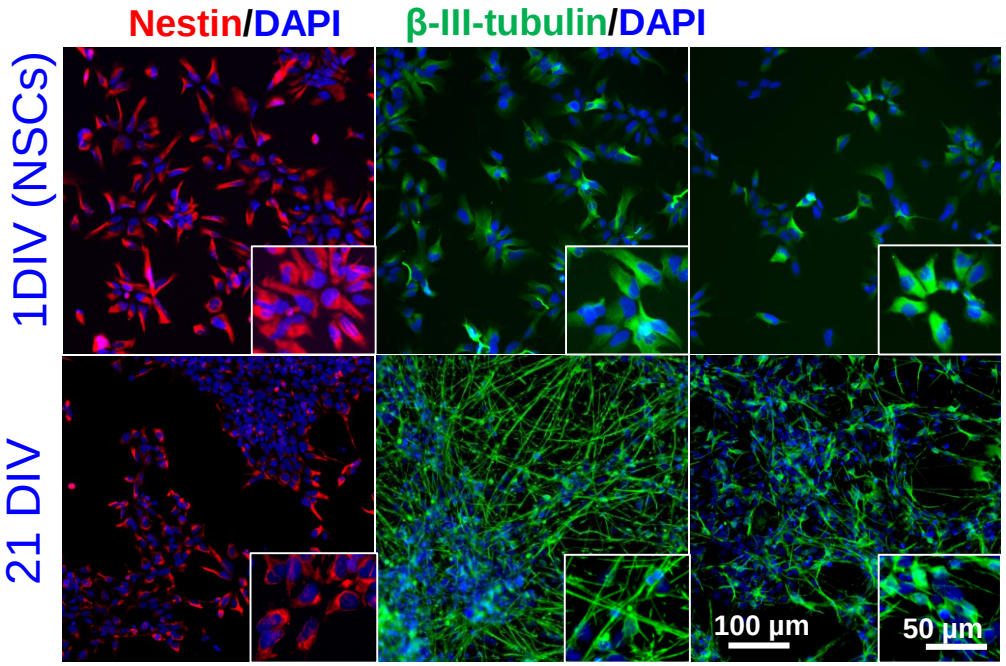


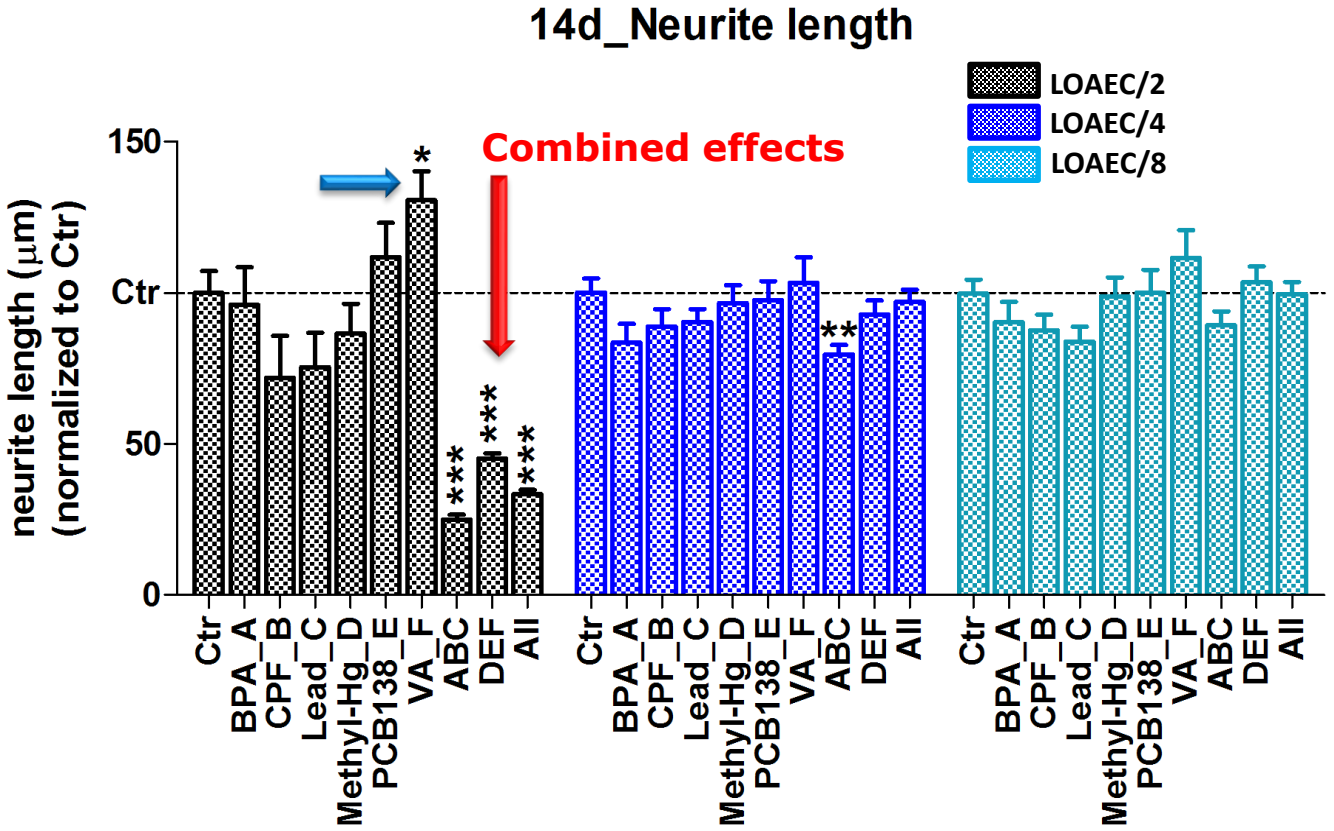
BPA	Chlorpyrifos	Lead (II)-Cl	Methyl -Hg	PCB138	Valproic Ac.	(μM)
12.0	21.0	0.007	0.0500	0.06	2.1	LOAEC
6.0	10.5	0.0035	0.025	0.03	1.05	LOAEC/2

Main (stat. signif.) effects:

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

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:

- Sim, Dis and ALL downregulates neurite length at LOAEC/2 and Sim already 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 "Dis" and "All"**)

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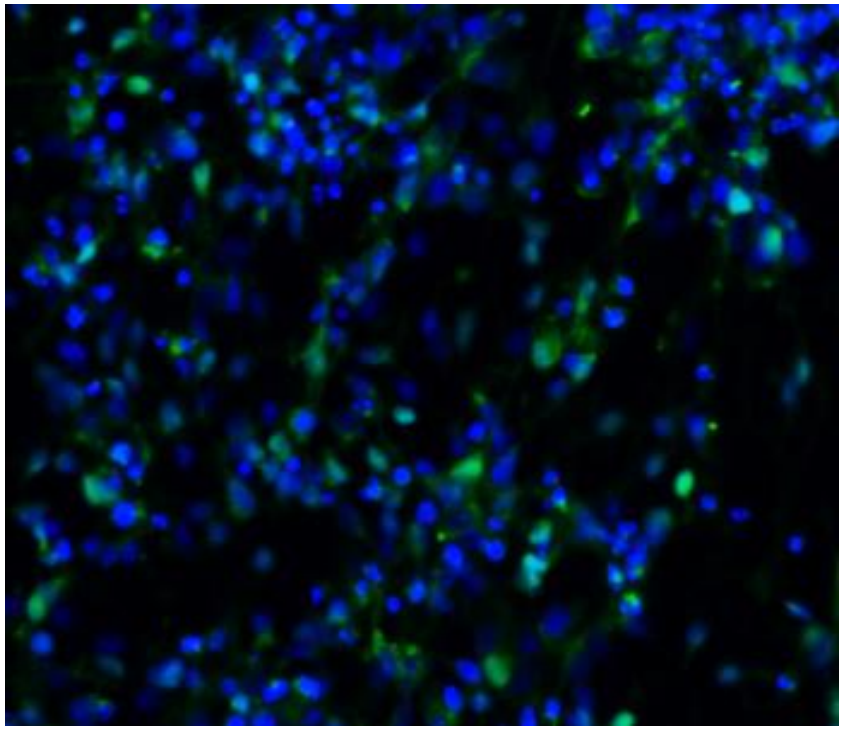
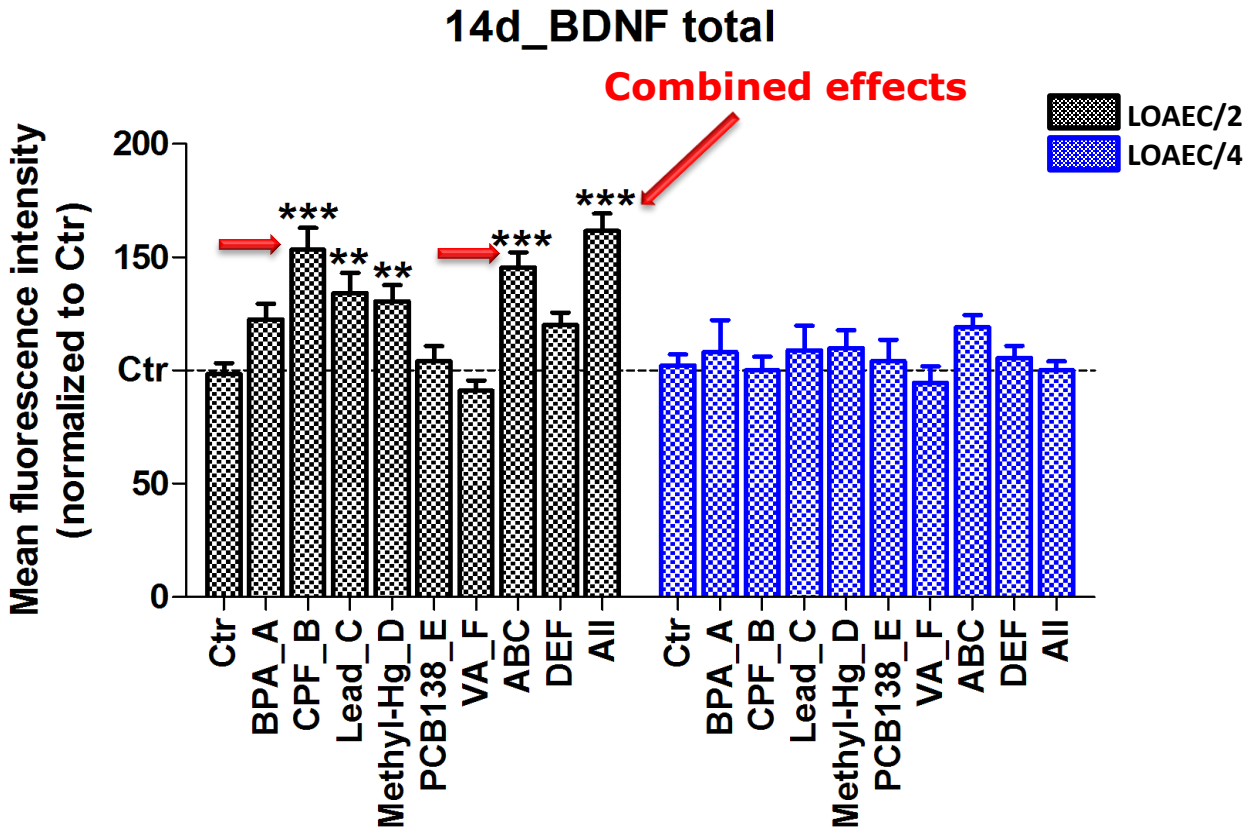
BDNF protein levels



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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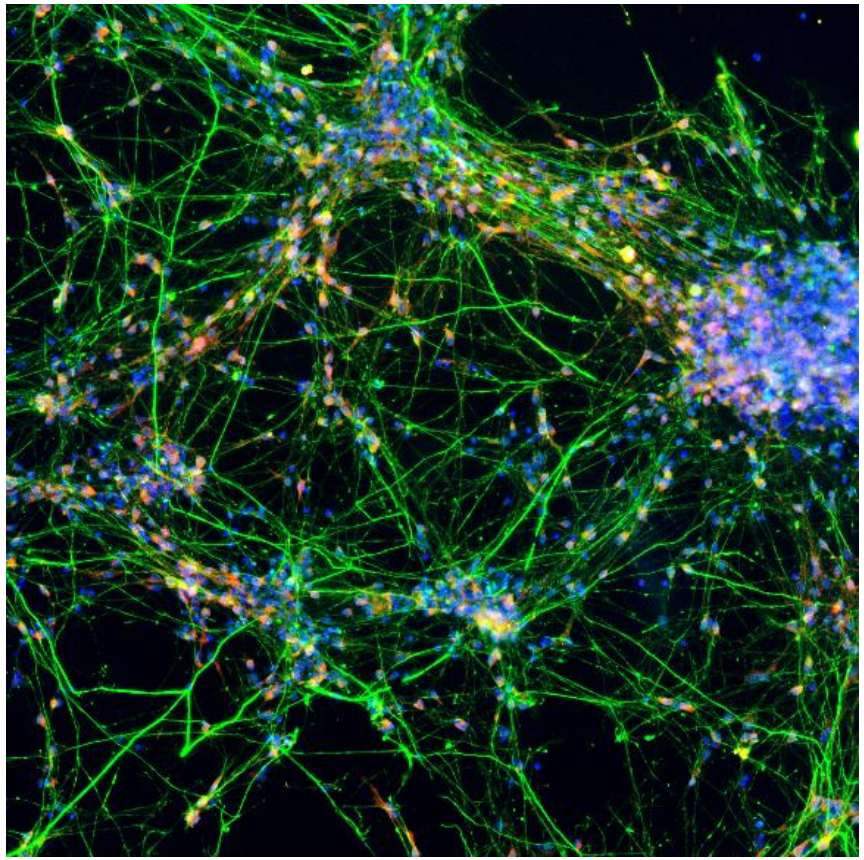
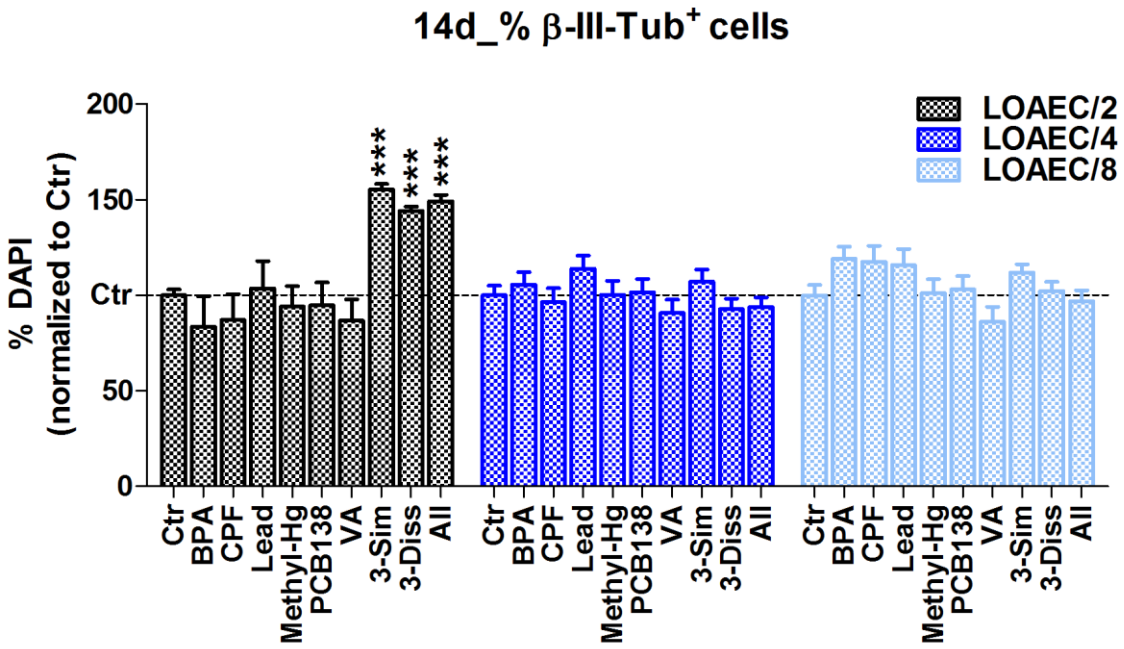
Neuronal cell percentage



GFAP/NF200/DAPI

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



BPA	Chlorpyrifos	Lead(II)-Cl	Methyl-Hg	PCB138	Valproic Ac	(μM)
6.4	37.0	0.7	0.06	6.0	210	LOAEC/2
3.2	18.5	0.35	0.03	3.0	105	LOAEC/4
1.6	9.25	0.175	0.015	1.5	52.5	LOAEC/8

Alterations 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)

- **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)

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)

Conclusions:

- Low concentrations (i.e., below LOAECs) of single chemicals (non-neurotoxic) become neurotoxic in mixture, especially for the chemicals working through similar MoA
- 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, etc.)
- Common Key Events identified in DNT AOPs guided selection of the in vitro assays, permitting mechanistic understanding of toxicity
- The obtained data will be used for refining the existing AOPs.

Assessment of developmental neurotoxicity induced by chemical mixtures using an adverse outcome pathway concept. *Pistollato F., Emilio Mendoza de Gyves, Stephanie K. Bopp, Carolina Nunes, Andrew Worth and Anna Bal-Price. Environmental Health, 2019 (under review).*

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

Different classes of chemicals at conc. **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

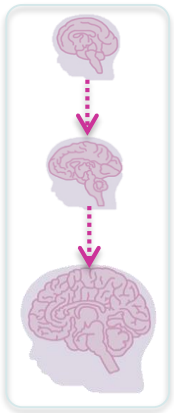
Non-toxic

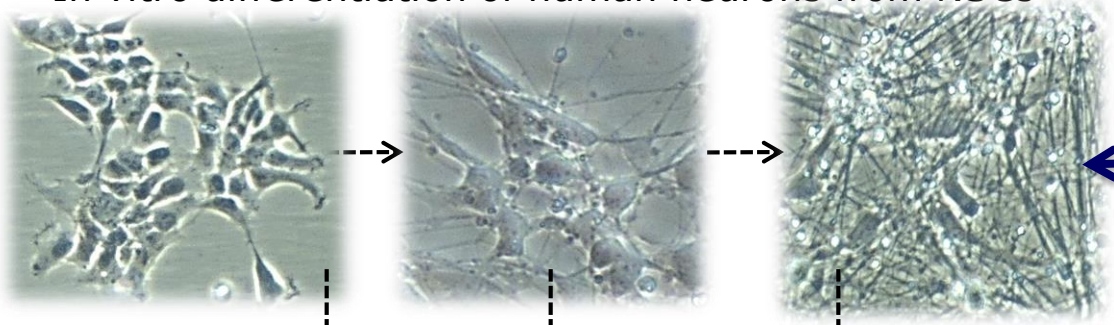
EXPOSURE (3 or 14 days)

single chemicals | mixtures

Toxic

In vitro differentiation of human neurons from NSCs





Differentiation of **human NSCs** towards neuronal and glial cells **represents key processes** critical for human brain development

In vitro assays anchored to **common KEs**

Altered BDNF levels | Altered neurite outgrowth | Altered synaptogenesis | Adverse outcome

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

Regulatory relevance

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

Acknowledgments

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Thank you for your attention!

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