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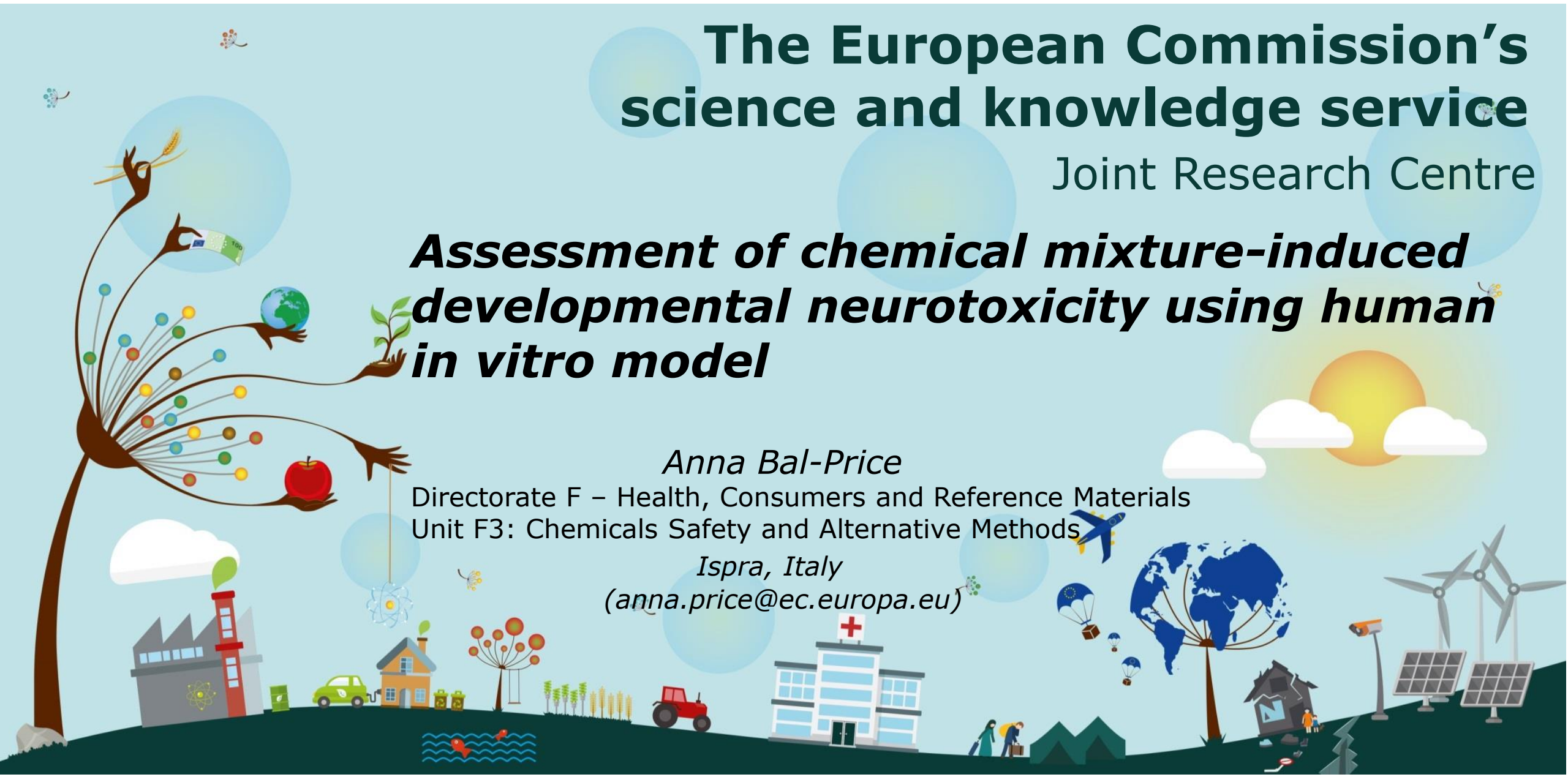

Assessment of chemical mixture-induced developmental neurotoxicity using human in vitro model

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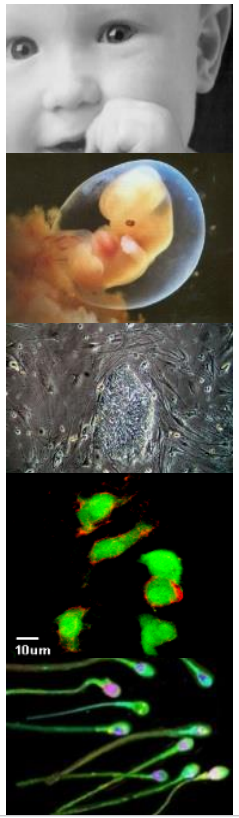
European Commission

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

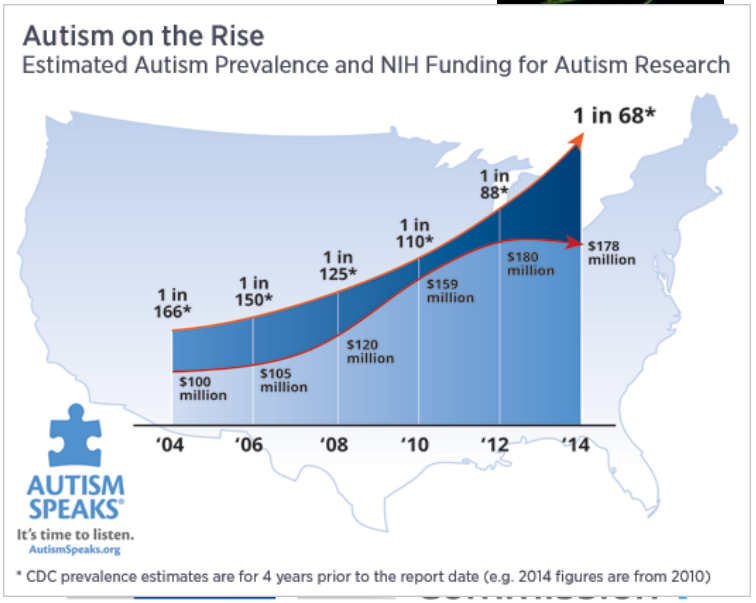


- **one in every six children** has a developmental disability that affect the nervous system *(Atladottir et al., 2015)*

- **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)*



Both laboratory (*in vivo* and *in vitro)* and human studies indicate that exposure to hazardous chemicals can contribute to DNT related effects.

Prime examples of chemical classes with potential to cause DNT effects:

- **Organophosphate and organochlorine pesticide** (e.g. chlorpyrifos, paraquat, DDT)
- **Combustion related air pollutants** (e.g. PAHs, nitrogen dioxide, particular matter)
- **Metals** (e.g. lead, mercury, cadmium, arsenic, manganese, triethyltin)
- **POPs** (PCBs, PBDEs flame retardants, perfluorate-PFOS and perfluorate-PFOA)
- **Organic solvents** (e.g. ethanol, toluene, xylene)
- **Drugs** (e.g. valproic acid, haloperidol, chlorpromazine, cocaine, dexamethasone)
- **Endocrine disruptors** (e.g. bisphenol A, perchlorate, triclosan, fluoride)

Existing DNT TGs are entirely based on *in vivo* studies

OECD

TG 426 DNT

EPA

OPPTS 8706300

EPA 712-C-98-239

FDA

EMEA

Safety neuro-pharmacology

ICH S7A (04.05 Washington)

DNT in vivo studies are triggered referring to evidence of neurotoxicity in standard systemic in vivo tests in adult animals or when data from extended one–generation reproductive toxicity study (TG 443) indicate a possible concern of neurotoxicity.

Toxicology and Applied Pharmacology 354 (2018) 3–6

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

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

Ellen Fritsche[a], Philippe Grandjean[b], Kevin M. Crofton[c], Michael Aschner[d], Alan Goldberg[e,w], Tuula Heinonen[f], Ellen V.S. Hessel[g], Helena T. Hogberg[h], Susanne Hougaard Bennekou[i], Pamela J. Lein[j], Marcel Leist[k], William R. Mundy[l], Martin Paparella[m], Aldert H. Piersma[n], Magdalini Sachana[o], Gabriele Schmuck[p], Roland Solecki[q], Andrea Terron[r], Florianne Monnet-Tschudi[s], Martin F. Wilks[t], Hilda Witters[u], Marie-Gabrielle Zurich[s], Anna Bal-Price[v,*]

ABSTRACT

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, SCAHT, Health Canada, JRC, FICAM, RIVM, Bayer 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:

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).

European Commission

Science of the Total Environment 543 (2016) 757–764

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Should the scope of human mixture risk assessment span legislative/regulatory silos for chemicals?

Richard M. Evans [a,*], Olwenn V. Martin [a], Michael Faust [b], Andreas Kortenkamp [a]

Box 1. Developmental neurotoxicants. Graphic shows four groups of chemicals identified as developmental neurotoxicants (Grandjean and Landrigan, 2014). Widths of coloured blocks are in proportion to the number of chemicals. The chemicals shown are subject to different pieces of legislation (Table 1), including pesticide residues (pesticides), REACH (industrial chemicals) and food contaminants (metals, PCBs). POPs are subject to a global treaty, the Stockholm Convention on Persistent Organic Pollutants, to which the EU are signatories.

(***Only one chance. How environmental pollution impairs brain development and how to protect brain of the next generation***. Grandjean P. New York, NY:Oxford University Press, 2013)

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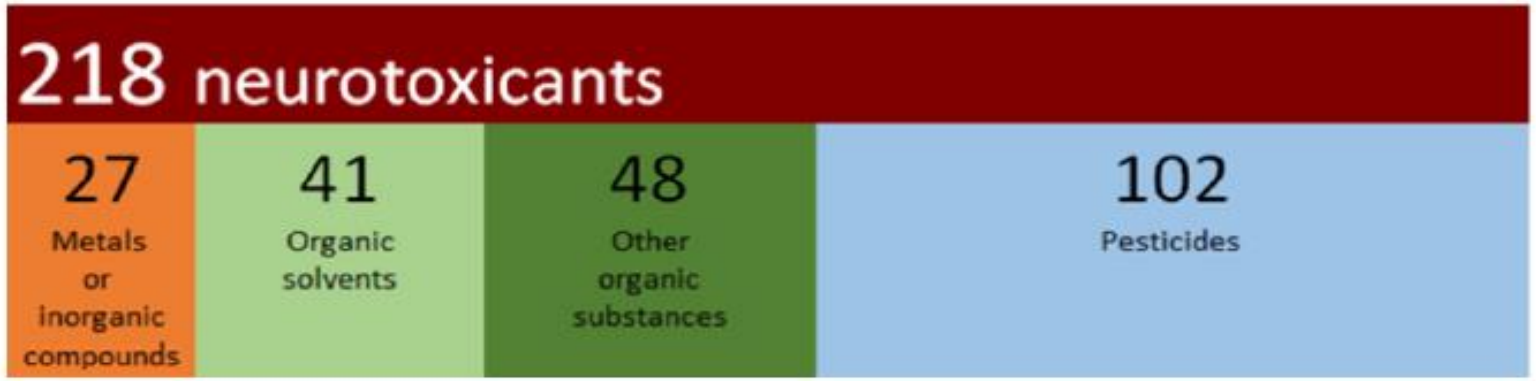
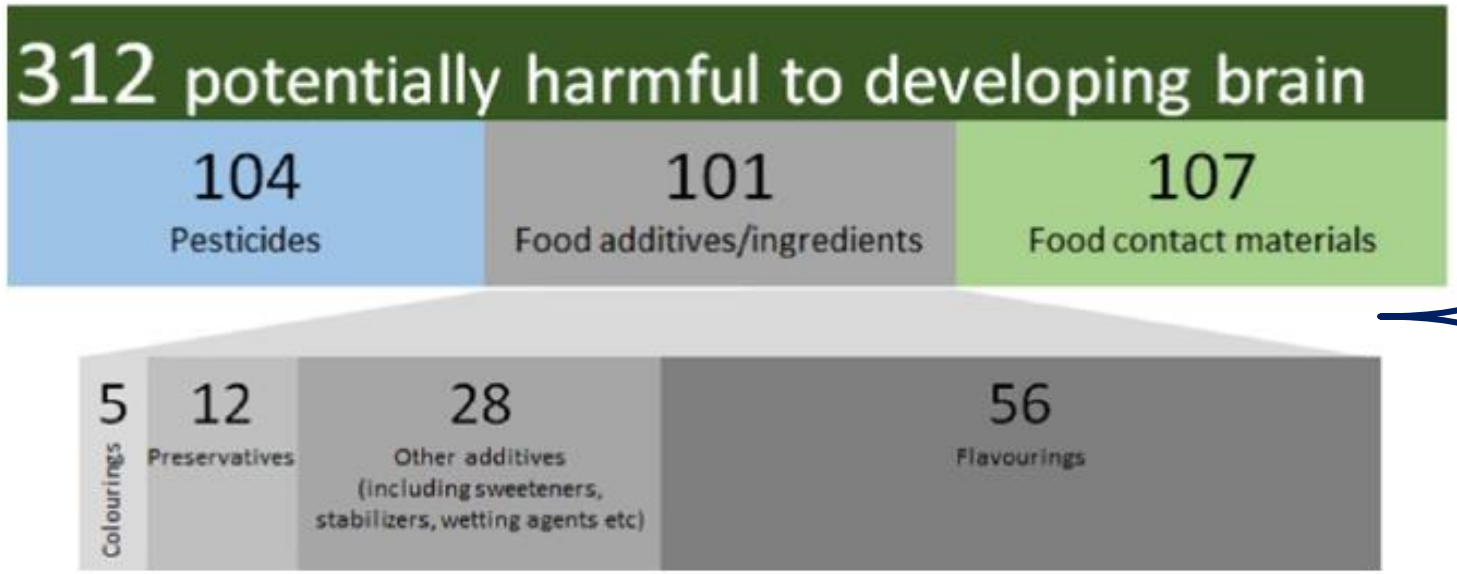


Box 2. Neurotoxicants. Graphic shows four groups of chemicals identified as neurotoxicants (Grandjean and Landrigan, 2014). Widths of coloured blocks are in proportion to the number of chemicals. The chemicals shown are subject to multiple pieces of legislation (Table 1), including REACH (industrial chemicals), food contaminants (metals) and pesticide residues (pesticides).



Chemicals grouped according to food related use

Box 3. Chemicals potentially harmful to the developing brain. Graphic shows 312 chemicals identified being potentially harmful to the developing brain, based on in vivo or in vitro evidence for effects on the brain or thyroid system, and grouped according to food-related use (Maffini and Neltner, 2015). Widths of coloured blocks are in proportion to the number of chemicals. The chemicals shown are subject to at least three different pieces of legislation (Table 1), including food contact materials, pesticide residues and food additives.

(Evans et al., 2016)

AOP-concept driven evaluation of DNT effects induced by mixture of chemicals

Aims of the study:

- Develop *in vitro* approach using human model relevant to brain development for mixture evaluation, covering different classes of chemicals (e.g., pesticides, industrial chemicals, heavy metals, polychlorinated biphenyls, endocrine disruptors, etc.).
- Build a battery of *in vitro* assays anchored to common key events identified in the network of existing DNT AOPs (*AOP-Wiki and Bal-Price et al., Crit. Rev. Toxicol., 2015,* 45(1):83-91).
- Select chemicals according to the established criteria.
- Define *LOAECs (Lowest Observable Adverse Effect Concentrations)* value for single chemicals and in mixture.
- Determine whether synergistic, antagonistic or additive effects are observed in mixture through mathematical modelling.

5.4.1.2. Grouping 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

Draft guidance on ***Harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals*** (EFSA Scientific Committee, 2018)

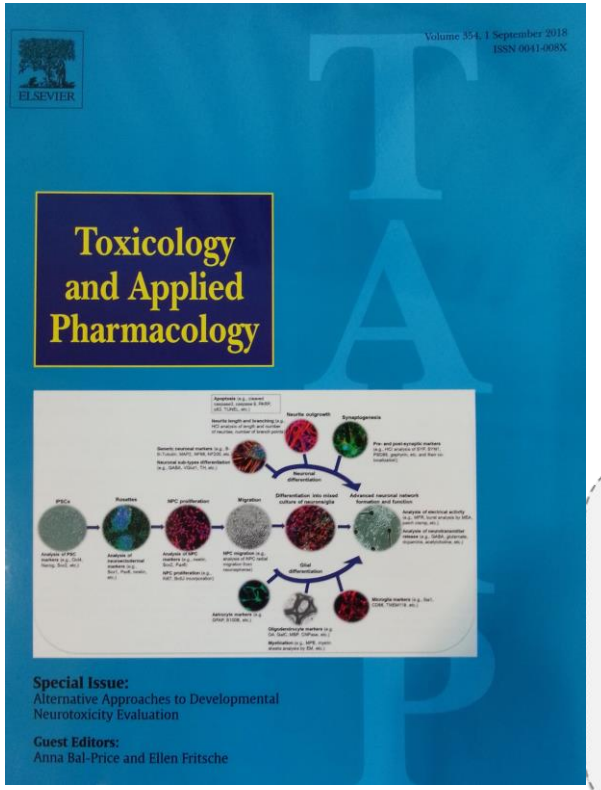
EFSA have recommended that pesticides which produce **common adverse outcomes on the same target organ/system (e.g. brain) should be grouped together for the purpose of assessing cumulative risk in relation to maximum residue limit (MRL) setting (EFSA, 2013).**

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

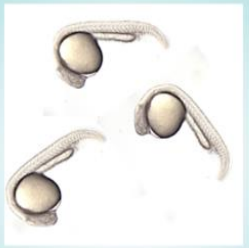
1. Inhibition of Na+/I- symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children (*AOP-Wiki, **JRC***);
2. Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental (*AOP-Wiki, EPA*)
3. Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals (*AOP-Wiki, EPA**, JRC***);
4. Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities (*AOP-Wiki, **JRC***);
5. Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging (*AOP-Wiki, University of Lausanne*)
6. The interaction of non-dioxin-like PCBs with ryanodine receptors (RyRs) causes their sensitization affecting neuronal connectivity that results in behavioral deficits (developmental neurotoxicity) (*Bal-Price et al., 2015, Crit Rev Toxicol*.)
7. Deficit in learning and cognition induced by exposure to mixture of metals As–Cd–Mn–Pb mediated by multiples MIEs (*von Stackelberg K., et al., 2015, Risk Anal.*)

Applied model: mixed neuronal/glial culture derived from human induced pluripotent stem cells (hiPSCs)

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, 354: 7-18, TAAP*



Non-mammalian models



Behavioural studies (e.g., with ZF embryos, or other non-mammalian species)

hiPSC-derived neuronal and glial models

Apoptosis (e.g., cleaved caspase3, caspase 9, PARP, p53, TUNEL, etc.)

Neurite length and branching (e.g., HCl analysis of length and number of neurites, number of branch points)

Neurite outgrowth

Synaptogenesis

Generic neuronal markers (e.g., B-III-Tubulin, MAP2, NF68, NF200, etc.)

Neuronal sub-types differentiation (e.g., GABA, VGlut1, TH, etc.)

Pre- and post-synaptic markers (e.g., HCl analysis of SYP, SYN1, PSD95, gephyrin, etc. and their co-localization)

Neuronal differentiation

iPSCs

Rosettes

NPC proliferation

Migration

Differentiation into mixed culture of neurons/glia

Advanced neuronal network formation and function

Analysis of PSC markers (e.g., Oct4, Nanog, Sox2, etc.)

Analysis of neuroectodermal markers (e.g., Sox1, Pax6, nestin, etc.)

Analysis of NPC markers (e.g., nestin, Sox2, Pax6)

NPC proliferation (e.g., Ki67, BrdU incorporation)

NPC migration (e.g., analysis of NPC radial migration from neurospheres)

Analysis of electrical activity (e.g., MFR, burst analysis by MEA, patch clamp, etc.)

Analysis of neurotransmitter release (e.g., GABA, glutamate, dopamine, acetylcholine, etc.)

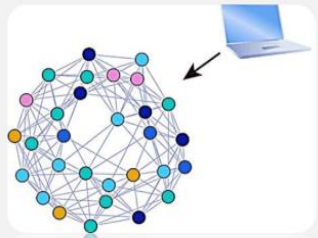
Glial differentiation

Astrocyte markers (e.g. GFAP, S100B, etc.)

Microglia markers (e.g., Iba1, CD68, TMEM119, etc.)

Oligodendrocyte markers (e.g. O4, GalC, MBP, CNPase, etc.)

Myelination (e.g., MPB, myelin sheets analysis by EM, etc.)



e.g., QSAR, read-across, IVIVE, computational modelling, etc.

***In silico* models**

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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, antiandrogenic)**
5	**TCDD**	**POP (ED, estrogenic)**

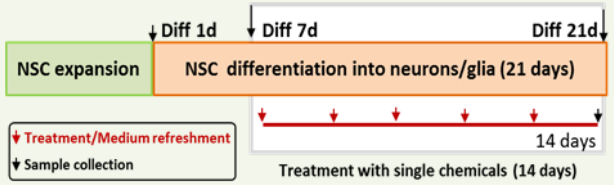
For instance, breast milk has been found to contain chemicals regulated as: **pesticides**; as **cosmetics**, including UV filters, parabens and phthalates; and as **persistent organic pollutants (POPs),** including polybrominated diphenyl ethers (PBDEs) and **polychlorinated biphenyls (PCBs)** (Schlumpf et al., 2010).

The experimental plan

Phase I

Evaluation of cytotoxicity for single chemical treatments

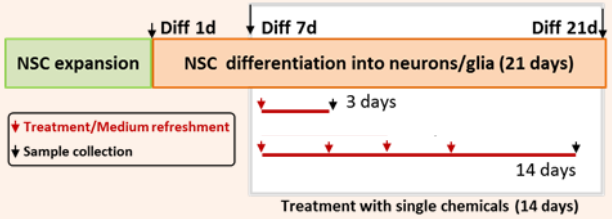
To define non-cytotoxic concentrations, low level toxicity ($IC_{20}/100$, IC_5), toxic (IC_{20}), with solvent control (0.1% DMSO)



(CellTiter Blue)

Phase II

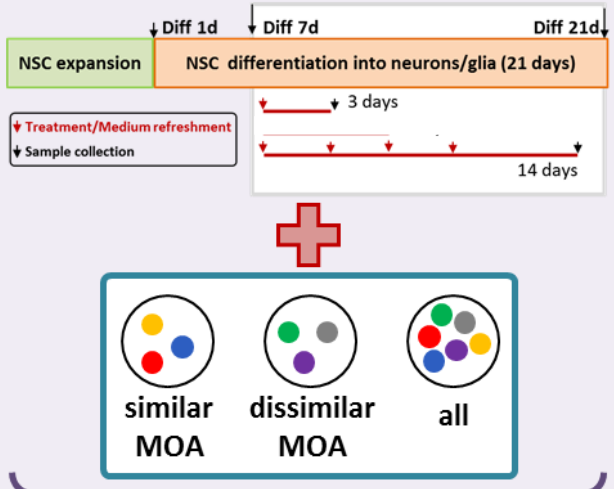
Repeated dose treatments with single compounds and analysis of DNT specific endpoints: synaptogenesis, neurites and BDNF expression



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

Phase III

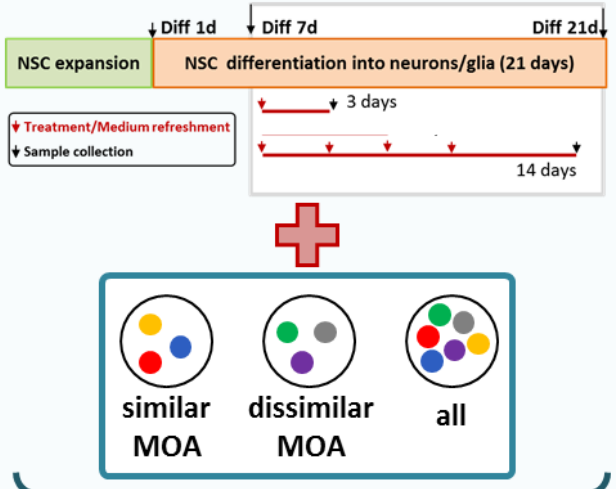
Repeated dose treatments with mixed compounds and analysis of DNT specific endpoints: synaptogenesis, neurites and BDNF expression



Additive
Synergistic
Antagonistic effects

Phase IV

Comparative analysis: mixed compounds at relevant exposure concentrations



Additive
Synergistic
Antagonistic effects

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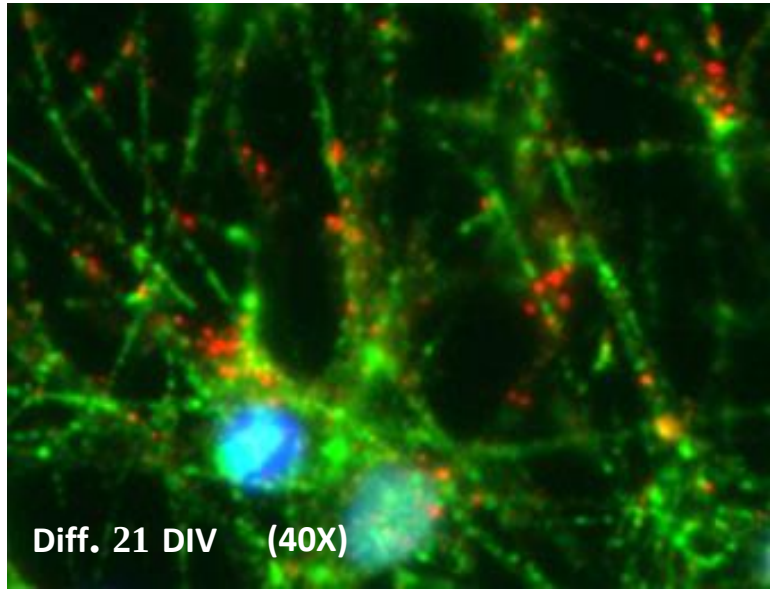
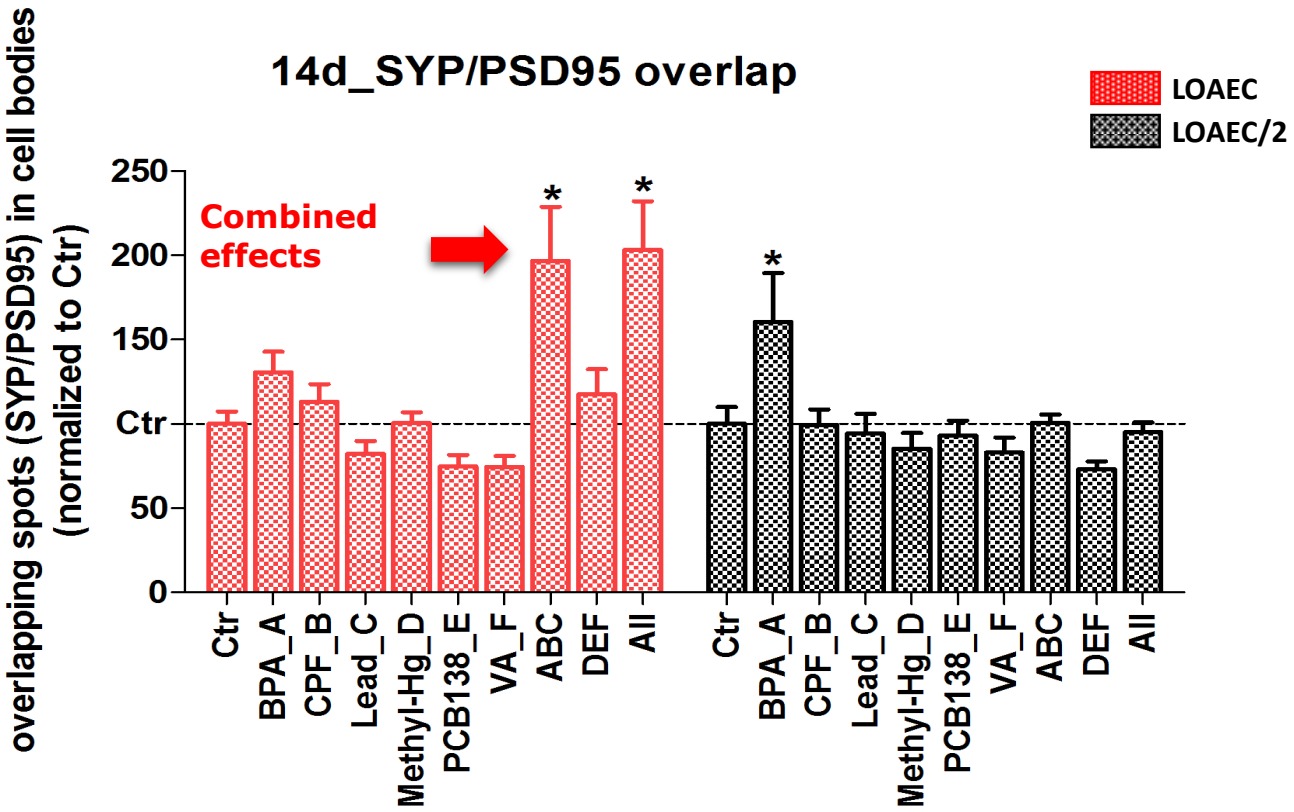
Phase III (some representative results)

Synaptogenesis (SYN)



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)

an
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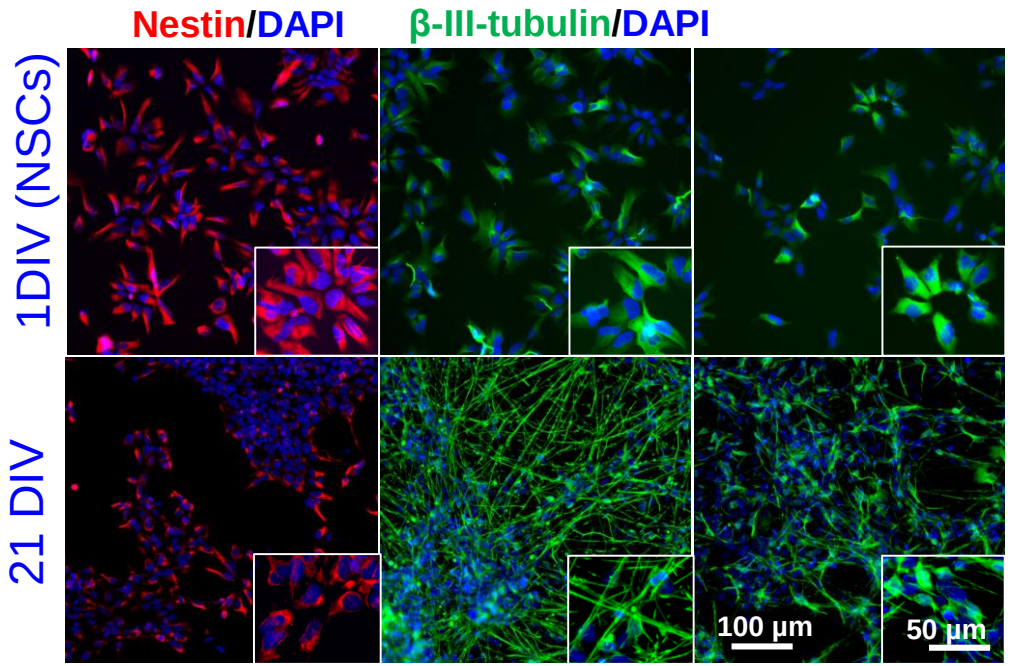
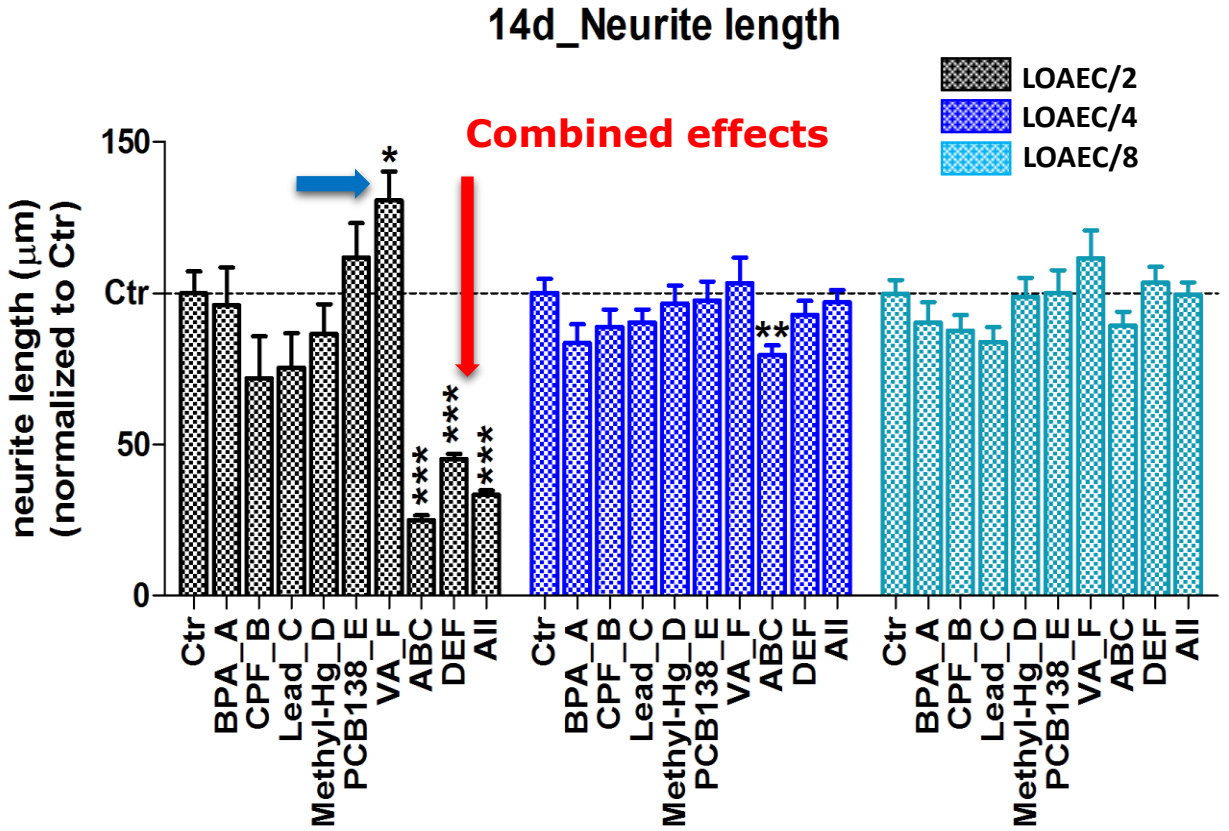
Neurite outgrowth





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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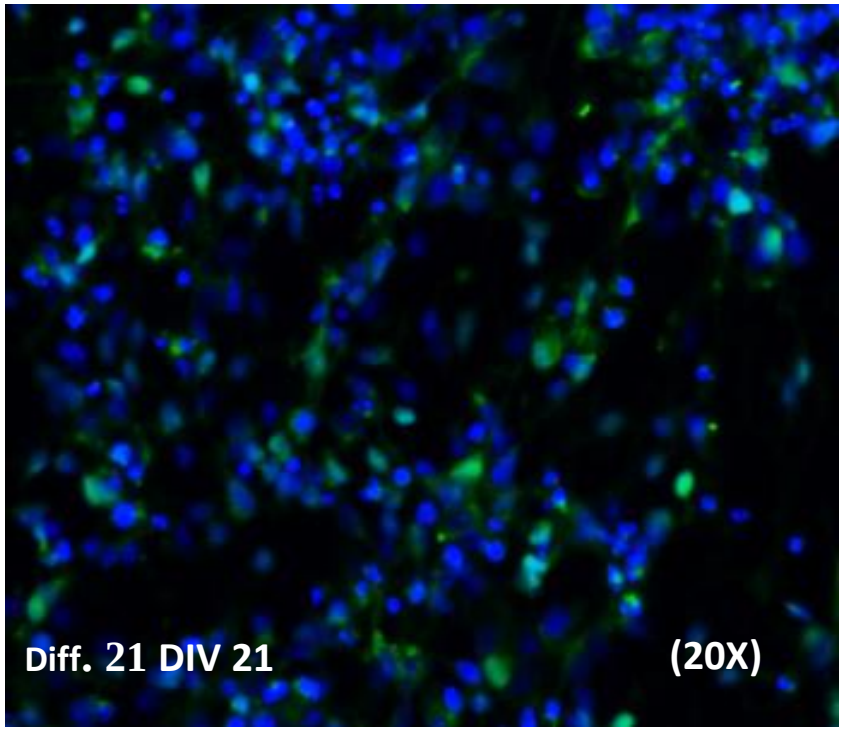
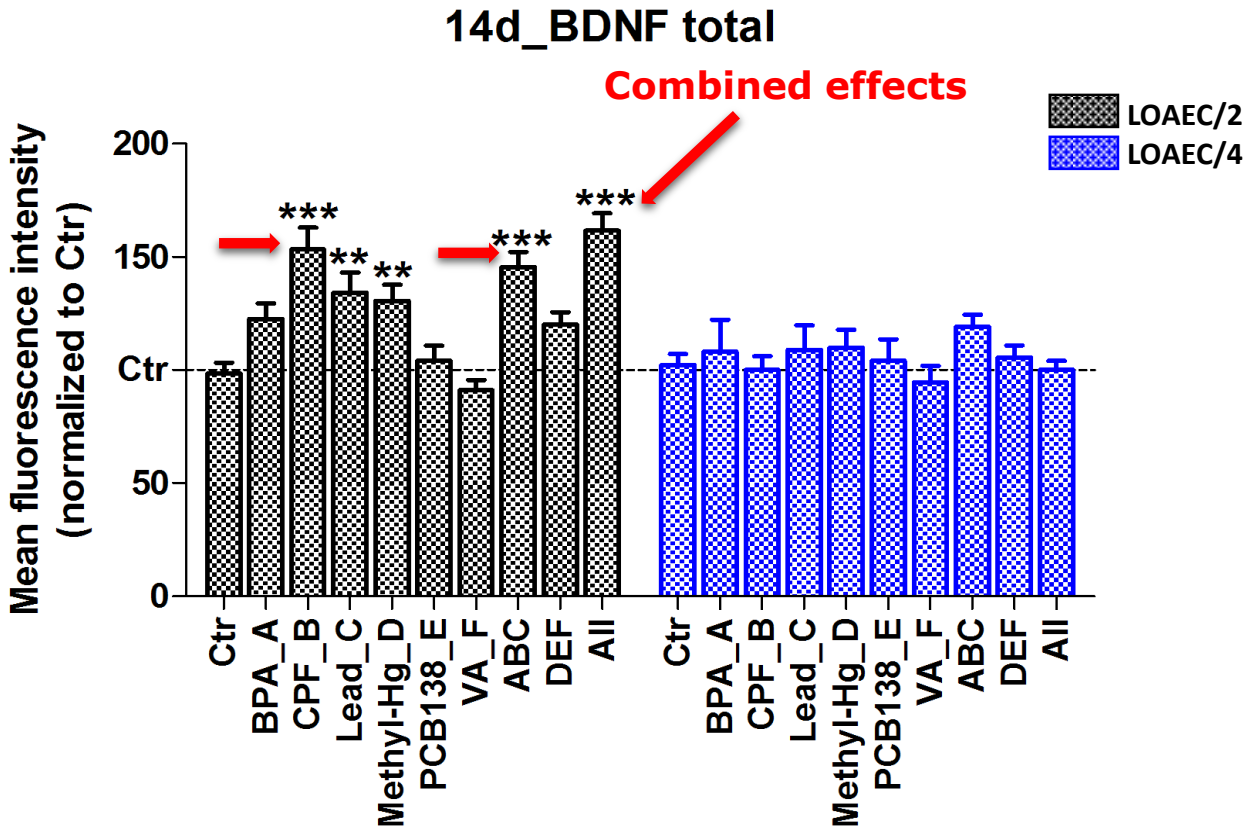
BDNF protein levels



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 (ongoing experiment: ABC and All without CPF)

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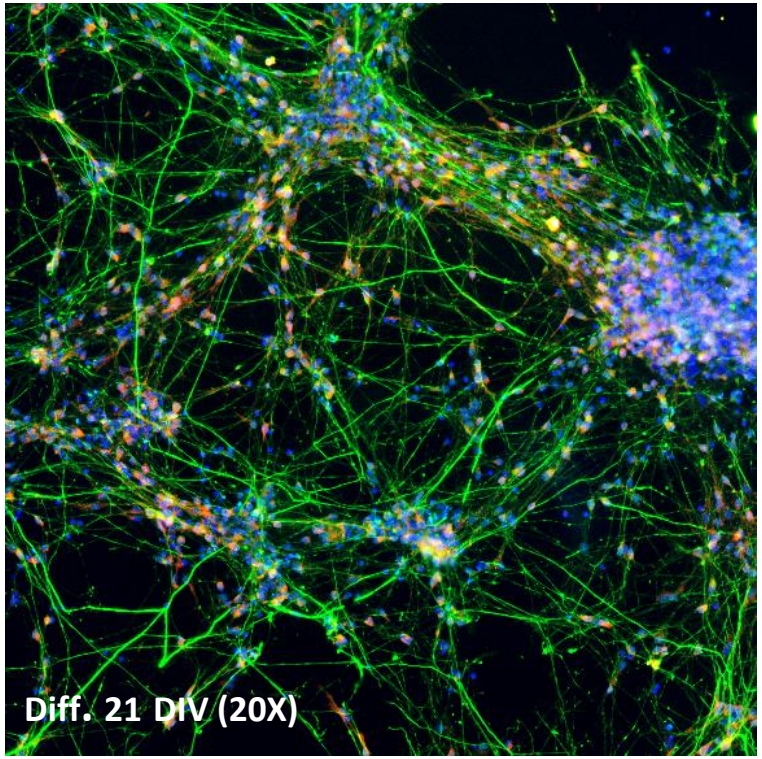
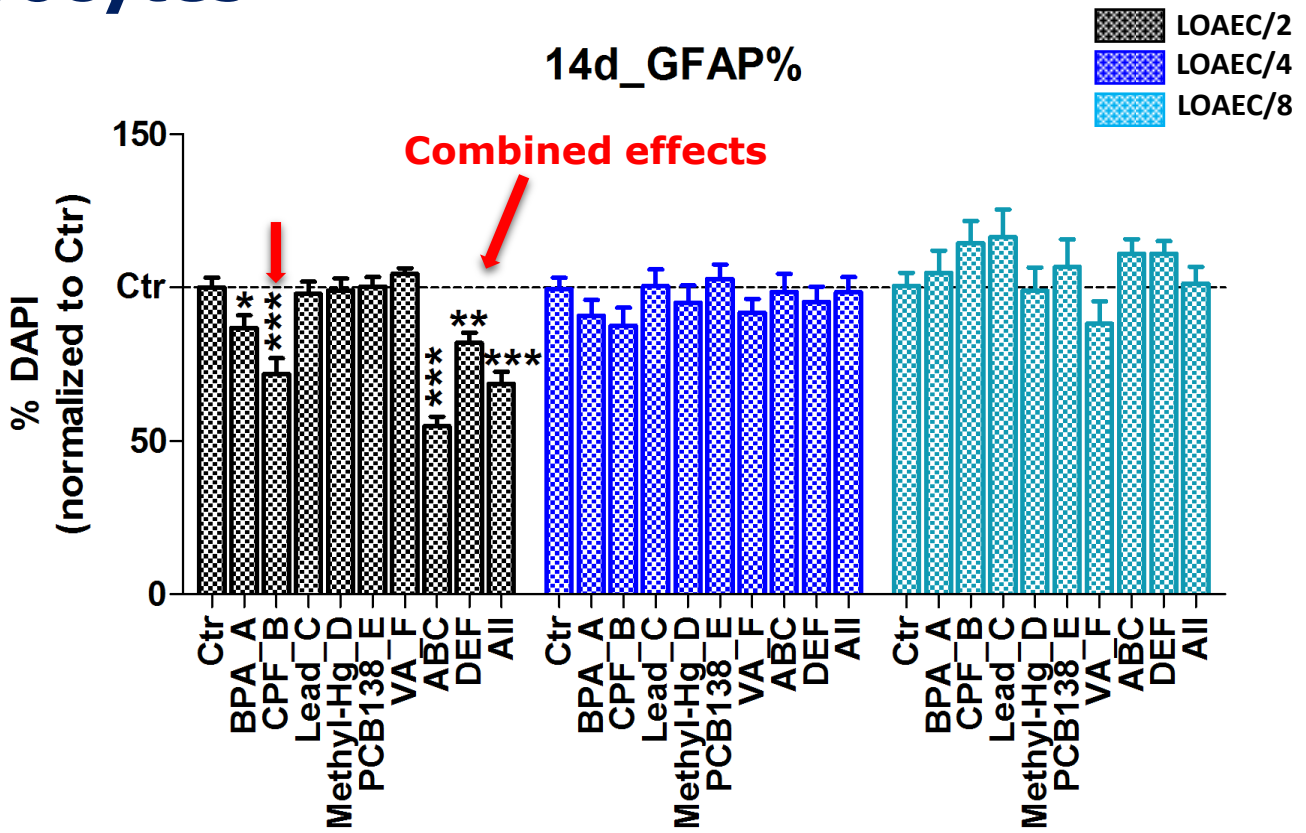
Presence of astrocytes



GFAP/NF200/DAPI



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

Main (stat. signif.) effects:

- CPF alone decreases GFAP+ cell percentage (at LOAEC/2)
- "ABC" (more than "DEF" and "All") decreases % of GFAP+ cells (at LOAEC/2)

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Conclusions:

- Our approach allowed to identify LOAECs for the studied single chemicals or in mixture
- Low concentrations (i.e., below LOAECs) of single chemicals (non-neurotoxic) become neurotoxic in mixture, especially for the chemicals working through similar MoA
- Combined effects of mixtures will be assessed by using the concept of dose addition to evaluate if they are additive, synergistic or antagonistic.
- Mixture toxicity of chemicals with similar MoA seems to be driven by chlorpyrifos.

- Mixed culture of neuronal and glial cells derived from human neural stem cells is a reliable *in vitro* model for studying DNT effects since it represents key processes critical for human brain development.

- DNT AOPs guided selection of the relevant *in vitro* DNT assays (e.g. anchoring them to identified common KEs), permitting mechanistic understanding of pathways involved.

- Networking of DNT AOPs provided valuable conceptual framework for evaluation of chemical mixture with potential to cause learning and memory impairment in children, one of the increasing concern of public health.

Acknowledgments

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European Commission

Thank you for your attention!

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