



Perspectives on how the Adverse Outcome Pathway concept informs the use of in vitro DNT data for regulatory purposes

Anna K. Bal-Price (anna.price@ec.europa.eu)

European Commission Joint Research Centre
Directorate F – Health, Consumers and Reference Materials
Unit F3: Chemicals Safety and Alternative Methods

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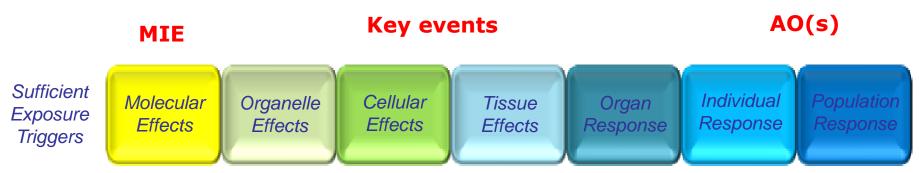
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Adverse Outcome Pathways - AOPs

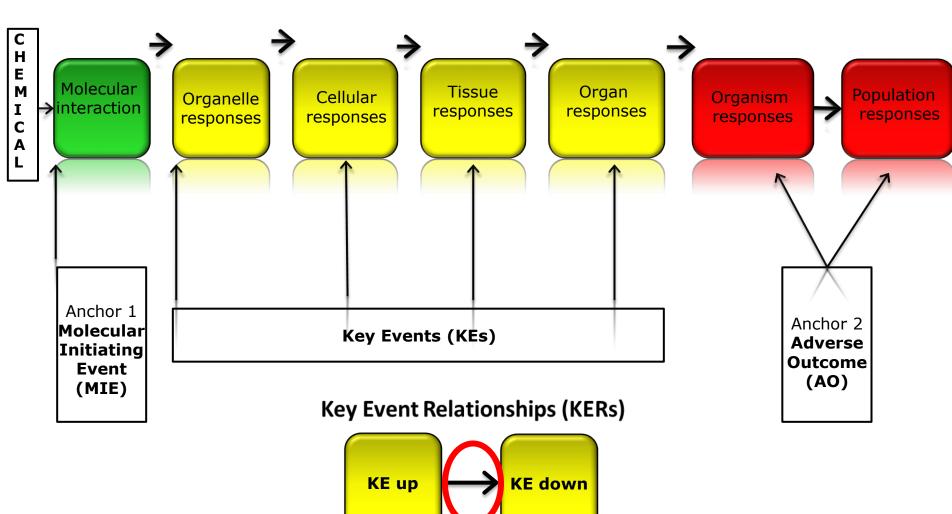
- Out of isolated Events a Pathway emerges:
 Adverse Outcome Pathway (AOP)
- An AOP is a conceptual framework that portrays <u>existing knowledge</u>
 between a Molecular Initiating Event and an **Adverse Outcome**
- An AOP is a mechanistic explanation of toxicity
- AOPs underpin the ongoing paradigm shift
 - away from observational black-box thinking toward predictive toxicology
- A focal point for is: collecting knowledge and assembling it into
 AOPs





Definition: An Adverse Outcome Pathway (AOP) is a conceptual framework that portrays existing knowledge concerning the linkage between a direct <u>molecular initiating event</u> and an <u>adverse outcome</u>, at a level of biological organization relevant to risk assessment. (Ankley et al. 2010, Environ. Toxicol. Chem., 29(3): 730-741.)





Assembling Weight of Evidence (WoE)



Modified Bradford-Hill Considerations	Conclusions	
Biological Plausibility	KER is consistent with current biological	
	understanding plausible.	
Essentiality of Key events	Effects are reversible if the stressor is removed (e.g., Villeneuve et al. 2009; EHP 117: 624-631)	
Concordance of Empirical Observations	Dose response – KEup occurs at lower doses than KEdown Temporality – KEup precedes KEdown Incidence – for a given dose, the incidence of KEup is greater than or equal to that of KEdown	
Consistency	Same pattern of effects has been observed in several tested species (e.g., fathead minnow, zebrafish, medaka)	
Analogy	Similar pattern of effects observed for known chemicals that belong to the same class 5	



AOPs are living documents

Operationally-defined "stages" of AOP development

	<u>. </u>	
Stages of AOP Development	Characteristics	Increasing Depth of evidence Understanding
Putative AOPs:	Hypothesized set of KEs and KERs primarily supported by biological plausibility and/or statistical inference	Transparency Defensibility
Formal AOPs (qualitative):	Include assembly and evaluation of the supporting weight of evidence – developed in AOP knowledgebase in accordance with internationally-harmonized OECD guidance	Quantitative precision
Quantitative AOPs:	Supported by quantitative relationships and/or computational models that allow quantitative translation of key event measurements into predicted probability or severity of adverse outcome	Cost Data needs Time

All stages have potential utility since level of AOP development required depends on the application.

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AOP applicability in regulatory context:

- AOPs (even qualitative) facilitate purpose-driven design and validation of in vitro methods (fit-for-purpose) based on identified MIEs and KEs (preferably CKEs)
- Data from high-throughput methods can represent a true 'first-tier' screen for the thousands of chemicals currently lacking data
- 2. Large data set produced by HTS platforms will serve as a base for development of predictive computational models
- 3. Chemical category formation:
 - grouping of chemicals with **similar structures** (MIE as profiler, QSAR models development
 - biological grouping according to triggered MIEs, KEs and AOs





4. Read-across: predicting unknown properties of one chemical from known properties of similar chemicals filling data gaps on the effects of chemicals by using AOP-mechanistic characteristics of the interaction between chemicals and the biological system (MIE) and biological responses (KEs).

Read-across can be significantly improved if reach data sets from HTS bioactivity vitro DNT assays are available.

- **5.** Hazard identification and hypothesis-driven testing: priority setting for further testing: pre-testing for a positive result or an alert, to minimize further animal testing.
- **6. Causative (or correlative) links between MIEs, KEs and AO based on** KERs empirical data and well documented biological plausibility should increase scientific confidence for regulatory use of *in vitro* DNT data. If information from multiple tests for the same pathway are consistent it should enhance the hazard assessment.
- 7. Risk assessment, if exposure and ADME data are available
- 8. Mechanistic understanding of pathways of toxicity in support of epidemiological studies (it verifies biological plausibility for an observed link between exposure and AO identified in epidemiological studies)

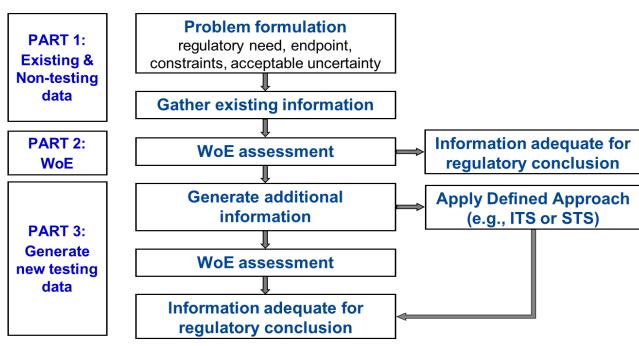


- **9. AOP provides a framework for** the discrimination of in vitro changes that are <u>adverse</u> (toxicologically relevant and predictive of the AO) from <u>those that are adaptive</u> (e.g. related to compensatory mechanisms that do not lead to AO).
- **10. Inter-species extrapolation:** conserved KEs within and across taxa enable identification of susceptible species
- **11. In vitro DNT data informs on conducting targeted in vivo testing** in a next step of a testing battery and improves interpretation of results derived from in vivo and guideline DNT testing.
- 12. AOPs facilitate testing of chemical mixtures:
 - a priori prediction of mixture effects on solid scientific knowledge base on identified KERs and AOs triggered by individual chemicals and linked to a network of AOPs.
 - In vitro data produced by the assays relevant to KERs identified in the DNT AOPs offer lower cost and HT of some endpoints, delivering hazard assessment of not only individual chemicals but also mixtures (difficult for in vivo studies: cost and logistics)
- **13. AOP provides conceptual framework for formulating defined approaches** (e.g. testing strategies), WoE, read across etc. within AOP-informed IATA (Integrated Approaches to Testing and Assessment)

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- it can guide selection of the most relevant DNT assays within IATA





- IATA is based on multiple information sources used for hazard identification, hazard characterisation and/or safety assessment of chemicals.
- ➤ IATA integrates and weights all relevant existing evidence and guides the targeted generation of new data where required to inform regulatory decisions. The overall assessment within IATA is performed on the basis of a non-formalised Weight of evidence.

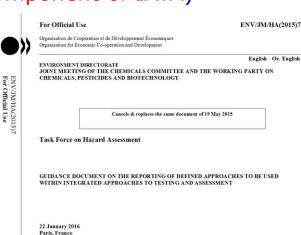


To facilitate IATA regulatory application *Defined Approaches* should be used within IATA for new data generation

- A defined approach to testing and assessment consists of a fixed data interpretation procedure (DIP) applied to data generated with a defined set of information sources (data coming from the most reliable, reproducible assays, used to build prediction model)
- The results can either be used on its own (fit-for-purpose), or together with other information sources within an IATA (DIP as a component of IATA)

OECD GD on the reporting of Defined Approaches to be used within IATA

- Led by the European Commission
- Proposed for adoption





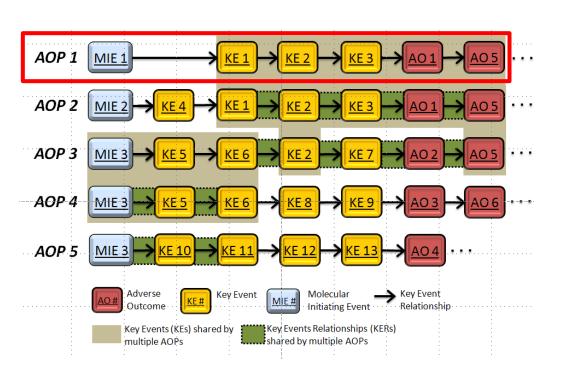


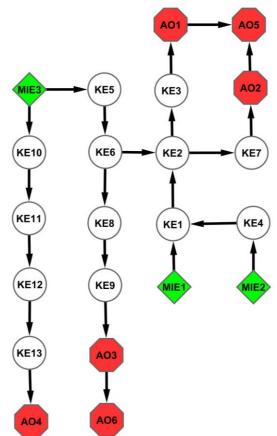
Could already available DNT AOPs serve as a guide for the selection of the most relevant in vitro assays for screening purposes to identify chemicals with DNT potential?

Network of AOPs



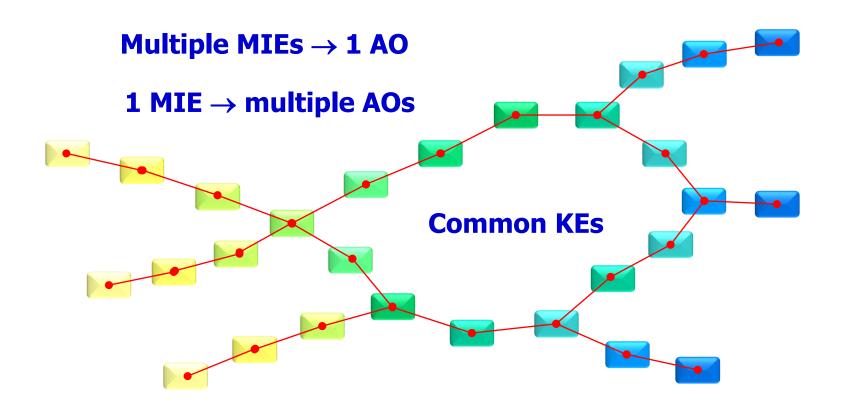
Each AOP is one sequential sequence or path through a broader network of AOPs.







DNT AOPs Networking





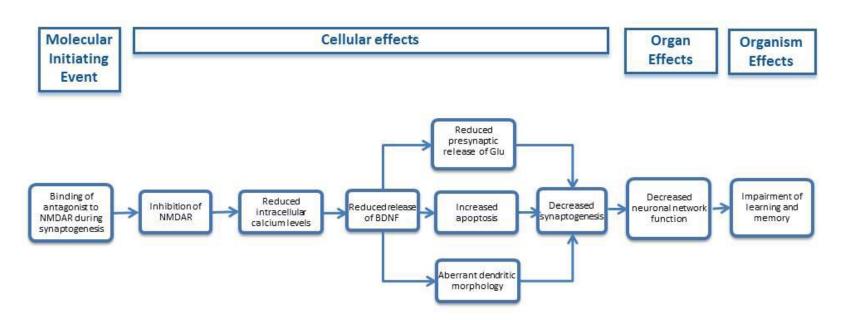
Networking of the current DNT AOPs that result in AO defined as impairment of learning and memory deficit/decrease cognitive function

(These AOPs are at different stages of evaluation and development)



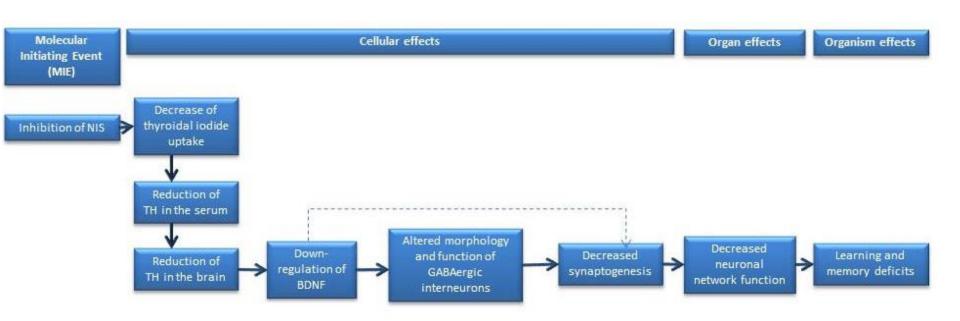
DNT AOPs that are triggered by various MIEs but all lead to cognitive/learning and memory impairment

AOP 1. Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities (*JRC*)





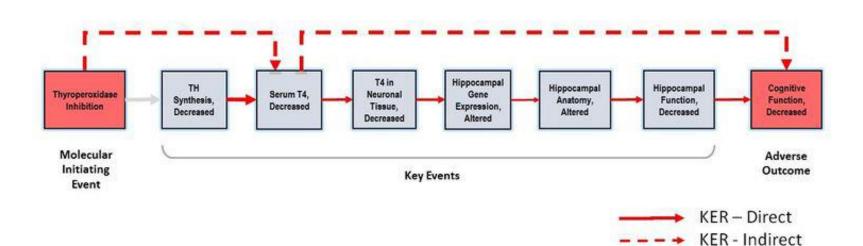
AOP 2: INHIBITION OF NA+/I- SYMPORTER (NIS) DECREASES THYRIOD HORMONE SYNTHESIS LEADING TO LEARNING AND MEMORY DEFICITS IN CHILDREN (*JRC*)



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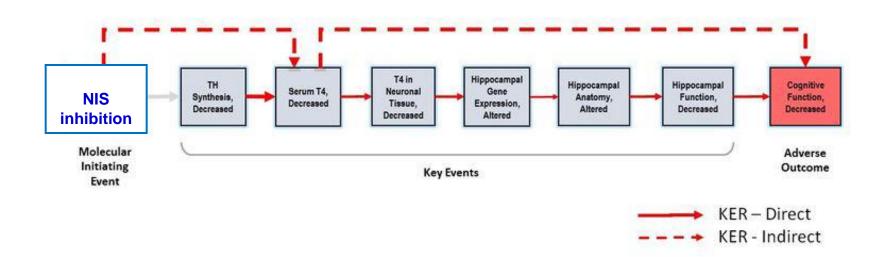
AOP 3: XENOBIOTIC INDUCED INHIBITION OF THYROPEROXIDASE AND SUBSEQUENT ADVERSE NEURODEVELOPMENTAL OUTCOMES IN MAMMALS (*US EPA*)







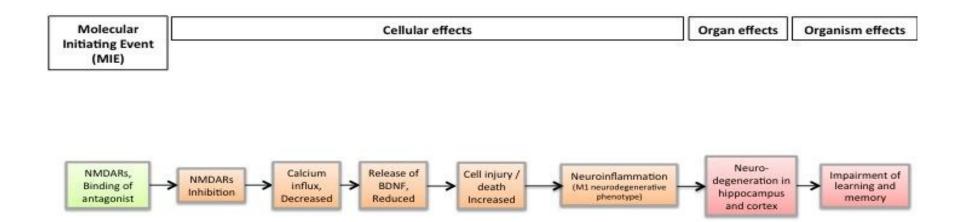
AOP 4: SODIUM IODINE SYMPORTER (NIS) INHIBITION AND SUBSEQUENT ADVERSE NEURODEVELOPMENTAL OUTCOMES IN MAMMALS (US EPA)





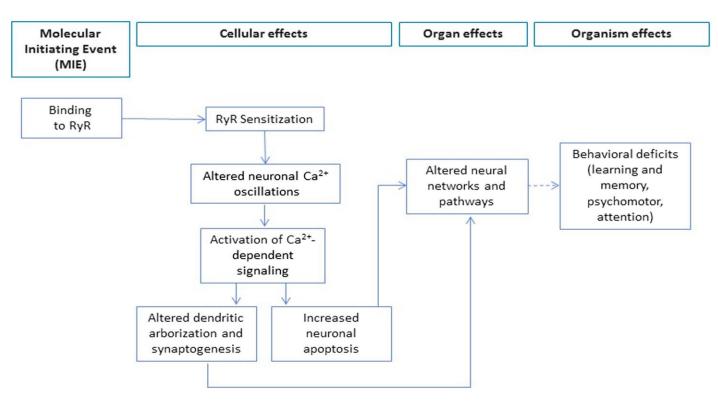


AOP5. Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration in aging (*Lausanne University*)





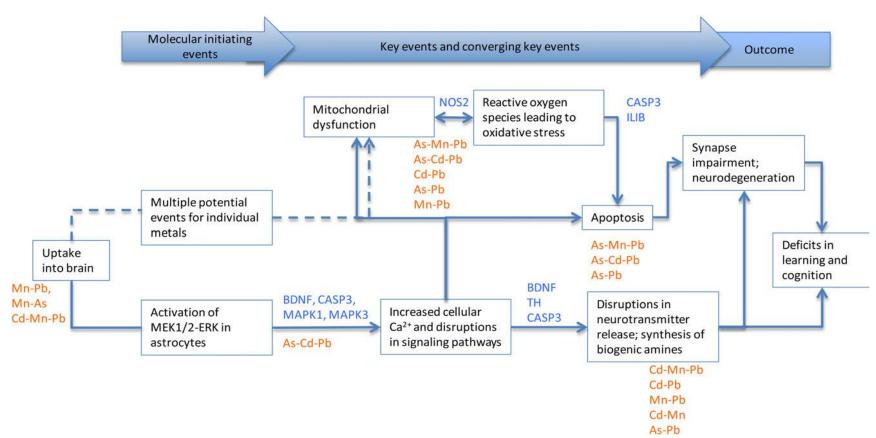
AOP 6: The interaction of non-dioxin-like PCBs with ryanodine receptors (RyRs) causes their sensitization affecting neuronal connectivity that results in behavioral deficits (developmental neurotoxicity) (*University California Davis*)



Bal-Price et al., Crit Rev Toxicol 2015



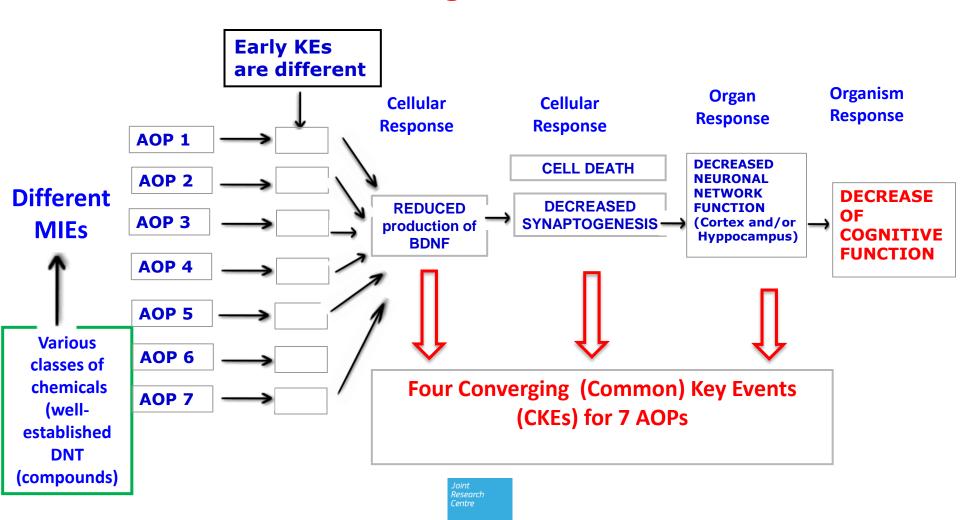
AOP7: Deficit in learning and cognition induced by exposure to mixture of metals As-Cd-Mn-Pb mediated by multiples MIEs



Exposure to Mixtures of Metals and Neurodevelopmental Outcomes: A Multidisciplinary Review Using an Adverse Outcome Pathway Framework. von Stackelberg et al., 2016, Risk Analysis.

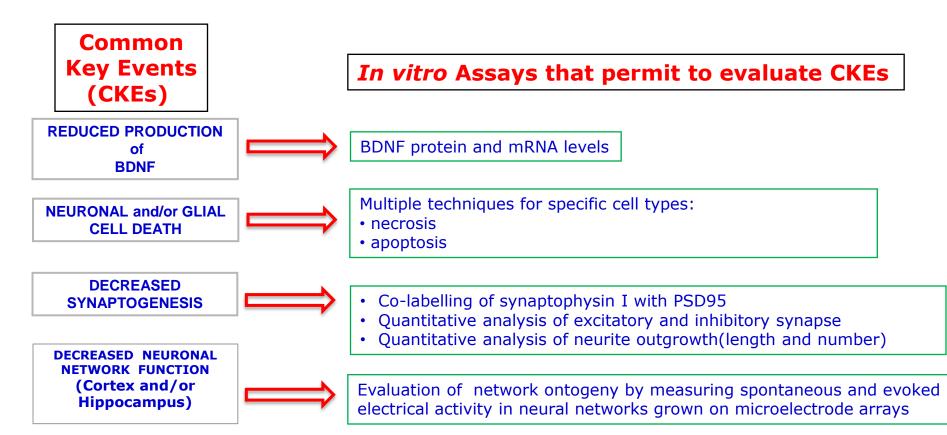


Seven DNT AOPs with multiple MIEs leading to the same AO: Decrease of Cognitive Function





Examples of a battery of *in vitro* assays for identification of chemicals with potential to decrease cognitive function based on 7 AOPs CKES



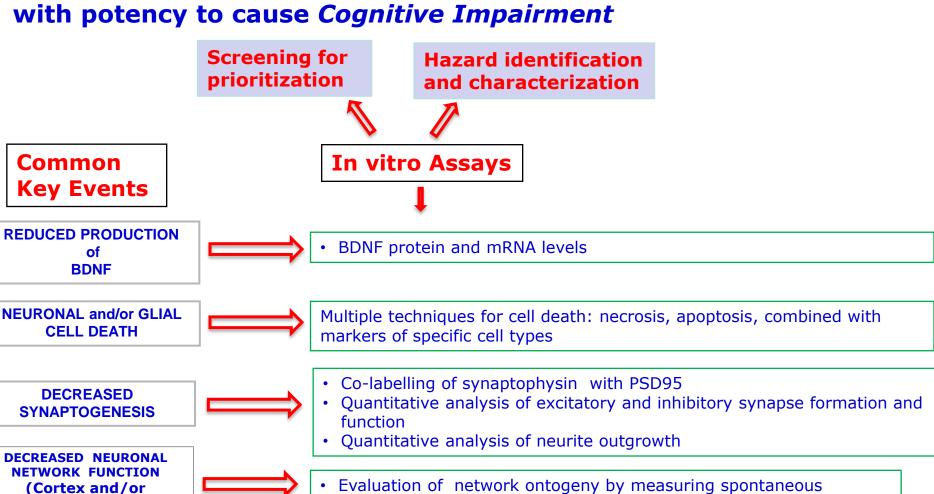


Currently available assays for measuring CKEs:

- 1. BDNF protein (sandwich ELISA kits, Western blotting, immuno-cytochemistry and immuno-fluorescence) and mRNA levels (e.g. RT-PCR, Northern blotting, DNA microarray etc.).
- 2. Multiple techniques for cell death combined with markers of specific cell types (necrosis: e.g. LDH release; propidium iodide etc; apoptosis (e.g. annexin V staining, TUNEL, caspases activation etc.)
- 4. Co-labelling of synaptophysin with PSD95 (HCA, e.g. Thermo Fisher Scientific kit).
- 5. Quantitative analysis of excitatory and inhibitory synapse formation and function (Cellomics, immuno-cytochemistry, HCA).
- 6. Quantitative analysis of neurite outgrowth (HCA and HTS: length, number etc.).
- 7. Evaluation of network ontogeny by measuring spontaneous or evoked electrical activity in neural networks (MEA, HCA).



Battery of assays anchored to AOPs CKEs to identify chemicals with potency to cause *Cognitive Impairment*



Hippocampus)

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electrical activity in neural networks grown on microelectrode arrays



AOP-informed IATA applicability in regulatory context:

Classification & labelling

Hazard identification

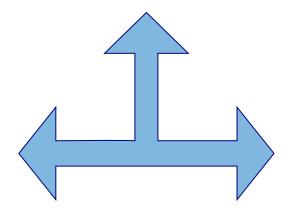
Integrated Approaches to Testing and Assessment (IATA)

Screening for priority setting

Risk assessment

Mechanistic information

- toxicokinetic pathways
- Adverse Outcome Pathways



Alternative Methods Toolbox

- •in vitro assays
- in silico models
- •in chemico assays
- Chemical categories

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Conclusions



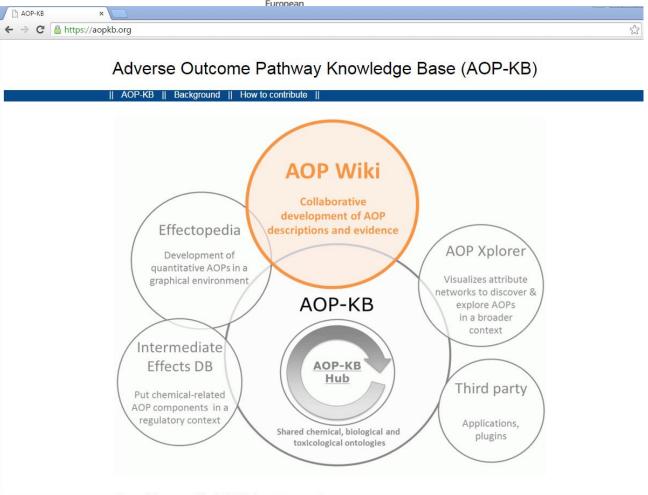
- 1. In vitro models and assays anchored to 7 AOPs CKEs are available to measure synaptogenesis and neuronal network formation and function: two key developmental processes for neuronal maturation evaluation
- 2. Screening chemicals through this battery of tests using HCA and HTS test methods will produce large data set.
- 3. Based on the obtained large data sets evaluate assays performance and select the test methods that are the most sensitive, reproducible and robust (assays and models scientific validation; consider utility of human iPSCs-derived mixed culture as first choice).
- 4. Combine the selected assays to create the final battery of tests to build *Defined Approaches* for a fixed data interpretation procedure (DIP) based on the established prediction model.



Next steps

- Further development of DNT AOPs taking into account the temporal and dose dynamics critical for neurodevelopmental processes and key DNT signalling pathways is necessary.
- 2. The **targeted generation of new quantitative data for KERs** to move DNT AOPs from being qualitative to quantitative.
- 3. **Combine assays anchored to KEs** in batteries of tests to produce data for fixed DIP.
- 4. Develop **DNT predictive models** based on large *in vitro* data set produced by the most reliable and reproducible HTS assays.
- 5. Build AOP-informed IATA(s) for defined, different regulatory purposes.





AOP Knowledge Base: https://aopkb.org/





Thank you for your attention!



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Recent publication with lists of DNT chemicals:

1. Expanding the test set: Chemicals with potential to disrupt mammalian brain development. Mundy WR, Padilla S, Breier JM, Crofton KM, Gilbert ME, Herr DW, Jensen KF, Radio NM, Raffaele KC, Schumacher K, Shafer TJ, Cowden J. Neurotoxicol Teratol. 2015 Nov-Dec;52(Pt A):25-35.

Approximately **100** developmental neurotoxicity test set chemicals were identified, with 22% having evidence in humans.

2. Neurite outgrowth in human induced pluripotent stem cell-derived neurons as a high-throughput screen for developmental neurotoxicity or neurotoxicity.

Ryan KR, Sirenko O, Parham F, Hsieh JH, Cromwell EF, Tice RR, Behl M. Neurotoxicology. 2016 Mar;53:271-81.

A library of **80** compounds screened for their ability to inhibit neurite outgrowth, in a high-throughput, high-content assay using human neurons derived from induced pluripotent stem cells (iPSC).

3. Quantitative assessment of neurite outgrowth in human embryonic stem cell-derived hN2 cells using automated high-content image analysis. Harrill JA¹, Freudenrich TM, Machacek DW, Stice SL, Mundy WR. . Neurotoxicology. 2010 Jun;31(3):277-90.

hN2 cells provide a model in which to investigate chemical effects on neurite outgrowth in a non-transformed human-derived cells and provide an alternative to the use of primary rodent neural cultures or immortalized clonal cell lines.





Multi-well microelectrode array recordings detect neuroactivity of ToxCast compounds. Valdivia P, Martin M, LeFew WR, Ross J, Houck KA, Shafer TJ.

Neurotoxicology. 2014 Sep;44:204-17.

105 chemicals tested: of the first set of 68 compounds (from the ToxCast Phase I and II libraries), 54 altered MFR by more than the threshold, while in the second set, 13/25 compounds were hits. MEAs detected 30 of 37 (81.1%) compounds that were hits in NVS_IC assays, as well as detected known neurotoxicants that were negative in ToxCast Novascreen assays, primarily pyrethroids and GABA receptor antagonists.

These data demonstrate that MEAs can be used to screen chemicals efficiently for potential DNT/neurotoxicity and that the results are concordant with predictions from ToxCast NVS_IC assays for interactions with ion channels.

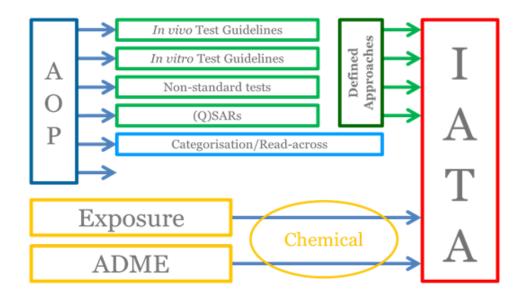
A multi-laboratory evaluation of microelectrode array-based measurements of neural network activity for acute neurotoxicity testing. Vassallo A, Chiappalone M, De Camargos Lopes R, Scelfo B, Novellino A, Defranchi E, Palosaari T, Weisschu T, Ramirez T, Martinoia S, Johnstone AF, Mack CM, Landsiedel R, Whelan M, Bal-Price A, Shafer TJ. Neurotoxicology. 2016 Mar 29. pii: S0161-813X(16)30041-9. doi: 10.1016/j.neuro.2016.03.019. [Epub ahead of print]

The data demonstrate that commercially available human iPSC-derived neurons are amenable for use in medium- to high-throughput assays using HCI and HTS. Though limited, the data indicate that human and rodent neurons can respond differently to chemical insult, and suggest that in vitro toxicity testing should include the use of human-derived cells.



AOP framework provides <u>a biological basis for IATA development</u>, which is a structured approach that strategically integrates and weights all kind of relevant data to inform regulatory decisions (**AOP-informed IATA**)

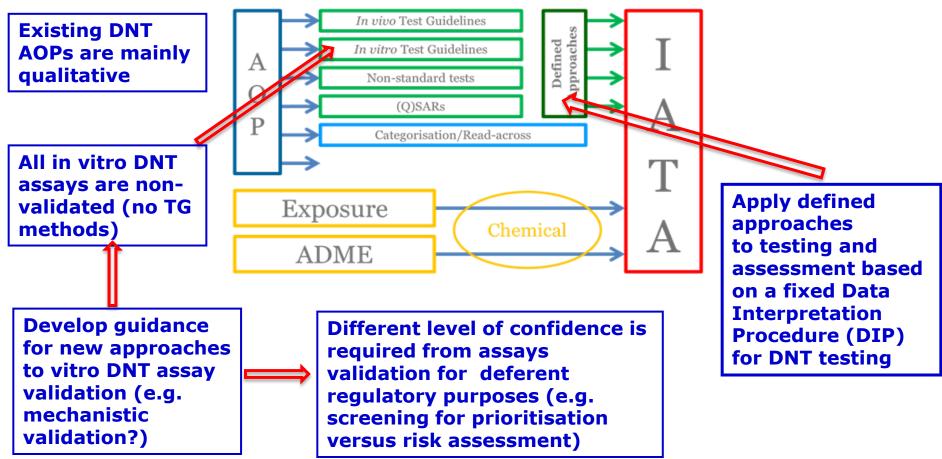
Elements within IATA







Elements within IATA



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AOP applicability in regulatory context

Relies on:

the maturity of the AOP (putative – qualitative - quantitative)

- the amount and type of supporting information
 - the confidence and precision of KEs measurements
 - the level of confidence in the KERs based on biological plausibility,
 empirical support and consistency of supporting data
 - the weight of evidence for the overall AOP
 - exposure and ADME data
 - prediction models

