Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

27-Jan-2017

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

Annex 2

REPORT OF THE OECD/EFSA WORKSHOP ON DEVELOPMENTAL NEUROTOXICITY (DNT): THE USE OF NON-ANIMAL TEST METHODS FOR REGULATORY PURPOSES

SLIDES OF SPEAKERS' PLENARY PRESENTATIONS
Series on Testing and Assessment
No. 261

Document produced by the Test Guidelines Programme work plan project 4.110, agreed in April 2016, and led by the European Food Safety Authority

Please note the deadline for declassification is 17 January 2017

JT03408278

ANNEX 5 – SLIDES OF SPEAKERS' PLENARY PRESENTATIONS

ENV/JM/MONO(2017)4/ANN2

[PPT 1] Why screening for developmental neurotoxicity? – A few thoughts from an epidemiological perspective

Thorhallur Halldorsson, University of Iceland on behalf of European Food Safety Authority (EFSA)

[PPT 2] Alternative Test Methods for Developmental Neurotoxicity: A History and Path Forward

Kevin Crofton, U.S. Environmental Protection Agency (USA)

[PPT 3] EU regulatory perspective with special focus on pesticides

Susanne Hougaard Bennekou, Danish EPA (DNK) and Roland Solecki, German Federal Institute for Risk Assessment (DEU)

[PPT 4] Developmental neurotoxicity under the REACH

Hannele Huuskonen, European Chemicals Agency (ECHA)

[PPT 5] US EPA's Regulatory Perspective on Developmental Neurotoxicity Studies: A Focus on Pesticides

Elissa Reaves, U.S. Environmental Protection Agency (USA)

[PPT 6] US Regulatory Perspective on Developmental Neurotoxicity Testing with Special Focus on Endocrine Disrupting and Industrial Chemicals

Stanley Barone Jr., U.S. Environmental Protection Agency (USA)

[PPT 7] EU Industry Perspective: Emphasis on Pesticides

Gaby Schmuck, representing European Crop Protection Association (BEL)

[PPT 8] U.S. Industry Perspective: DNT Testing Strategies Based on Alternative Assays

Sue Marty, DOW (USA)

[PPT 9] Perspectives on how the Adverse Outcome Pathway concept informs the use of in vitro DNT data for regulatory purposes

Anna Price, European Commission Joint Research Centre (JRC)

[PPT 10] How to link test systems to the prediction of developmental neurotoxicity (DNT)

Marcel Leist, University of Kostanz (DEU)

[PPT 11] Introduction to OECD case studies for potential testing strategies and a draft framework for building a DNT testing battery

Ellen Fritsche, Dusseldorf University (DEU)

[PPT 1] Why screening for developmental neurotoxicity? – A few thoughts from an epidemiological perspective

Thorhallur Halldorsson, University of Iceland on behalf of European Food Safety Authority (EFSA)

Why screening for developmental neurotoxicity? – A few thoughts from an epidemiological perspective

Þórhallur Ingi Halldórsson
University of Iceland
on behalf of the European Food Safety Authority (EFSA)

(tih@hi.is or lur@ssi.dk)

One reason why are we here

Grandjean P and Landrigan PJ *Lancet 2006*

Developmental neurotoxicity of industrial chemicals

P Grandjean, PJ Landrigan

Neurodevelopmental disorders such as autism, attention deficit disorder, mental retardation, and cerebral palsy are common, costly, and can cause lifelong disability. Their causes are mostly unknown. A few industrial chemicals (eg,

Lancet 2006; 368: 2167–78
Published Online

"Exposure during fetal development may cause brain injury at doses much lower than those affecting adults …."

…. still only few chemical are recognized as neurotoxic"

Recognized chemicals …

They identified 5 chemicals in 2006 (*known knowns*):

- **Lead**
- **Methyl-mercury**
- Arsenic
- Polychlorinated biphenyls (PCBs)
- Toluene

- … and a long list of *known unknowns (n>1000)*.

- … and *unknown unknowns (n>80000) !!*

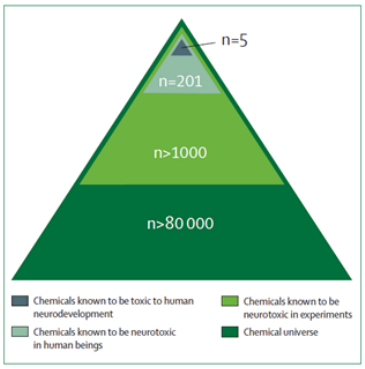


Figure 2: **Diagram of the extent of knowledge of neurotoxic chemicals**
Of the thousands of known chemicals, only a small fraction have been proven to cause developmental neurotoxicity in humans. Although this evidence does not represent the true potential for industrial chemicals to cause neurodevelopmental disorders, assessments of need for preventive measures nonetheless rely on that information.

Identification of chemicals follows a pattern which has a lot to do with dose

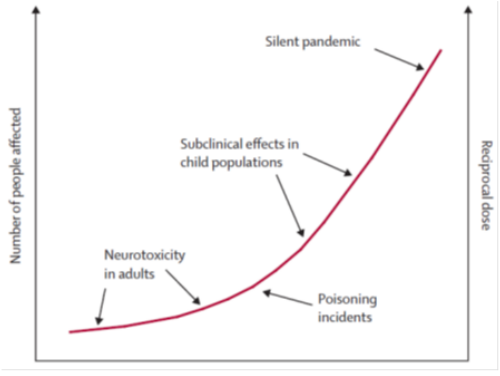


Figure 1: **The effects of a neurotoxic chemical on a population over time**
For identification of chemicals toxic to neurodevelopment, the first evidence dealt with adverse effects of high doses on the adult nervous system, and was followed by case reports and epidemiological evidence on developmental toxicity at successively lower doses, to which childhood populations of increasing magnitude were exposed. Recognition of inorganic lead, methylmercury, and polychlorinated biphenyls as neurotoxic followed this curve

Early-life exposures

Short and long term consequences

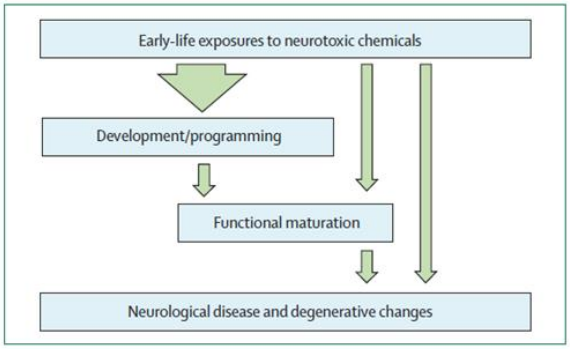


Figure 1: **Effect of neurotoxicants during early brain development**
Exposures in early life to neurotoxic chemicals can cause a wide range of adverse effects on brain development and maturation that can manifest as functional impairments or disease at any point in the human lifespan, from early infancy to very old age.

And a few years later they added more chemicals

Grandjean P and Landrigan PJ *Lancet Neurol 2014*

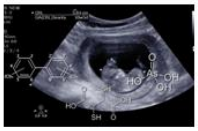
Neurobehavioural effects of developmental toxicity

Philippe Grandjean, Philip J Landrigan

Lancet Neurol 2014; 13: 330–38 Neurodevelopmental disabilities, including autism, attention-deficit hyperactivity disorder, dyslexia, and other

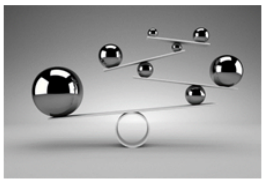
- Manganese
- fluoride,
- chlorpyrifos,
- Dichlorodiphenyltrichloroethan
- Tetrachloroethylene,
- polybrominated diphenyl ethers

BUT …… (there's always a but)



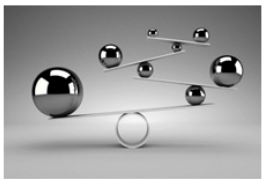
Lack of balance!

- For some of the compounds identified, many of the epidemiological studies were of low quality
- Non-confirmative studies were often not cited and ignored
- Uncertainty not given much weight



Lack of balance!

- For some of the compounds identified, many of the epidemiological studies were of low quality
- Non-confirmative studies were not often no cited and ignored
- Uncertenty not given much weight
- **But they are making a valid point ....**
- **And regardless of how we weight the evidence some of the facts cannot be ignored**



Perinatal exposures to Pb

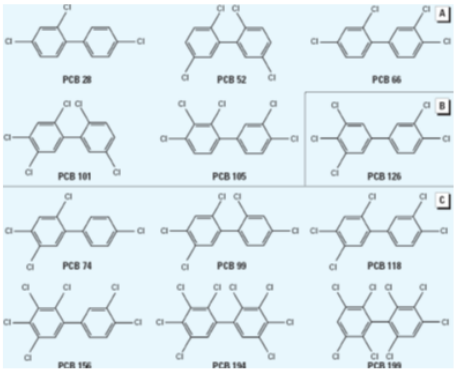
- IQ, concentration, memory, cognition, and behavior
  (Landrigan PJ, 1975; Needleman HL, 1979)
- Even at low concentrations (<10μg/dL)
  (Lanphear BP et al EHP 2005)
- For compounds that can easily be quantified and concentration reflect exposure over reasonable time ....
- ...it is not difficult to evaluate long term consequences of developmental exposures (even at low concentrations)
- ....it just takes time

Same goes for methyl-mercury
(also a question of dose)

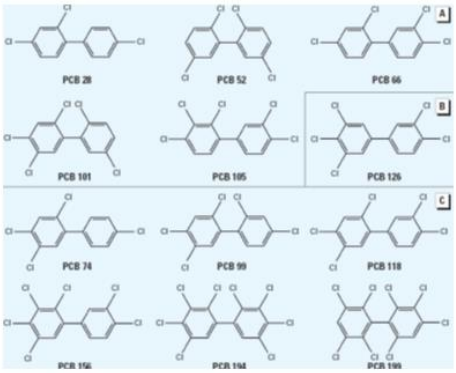
- High exposures (Minamata 1960)
  - profound mental retardation *(Harada M, 1995)*.
- Seafood, particularly predator fishes:
  - impairment in memory, attention, language, and visuospatial perception documented in children *(Grandjean P, 1997)*.
- "Normal Seafood"
  - Endless discussion of benefits and risk of fish consumption (as we approach "zero")

For organic chemicals the picture is more complex

- Even for persitent chemicals such as the PCBs quantifaying exposure is complex.
- Divergent findings on neurotxicity



For organic chemicals the picture is more complex



- For non-persitent compouds obtaining a reliable exposure estimates is very challenging

What about pecicides (OPs) - a more balanced review-

Toxicology Letters 230 (2014) 104–121

Contents lists available at ScienceDirect

Toxicology Letters

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

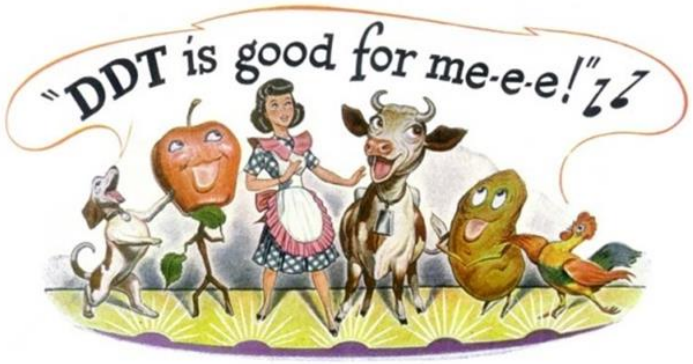
A systematic review of neurodevelopmental effects of prenatal and postnatal organophosphate pesticide exposure

B. González-Alzaga[a], M. Lacasaña[a,b,*], C. Aguilar-Garduño[c], M. Rodríguez-Barranco[a,b], F. Ballester[b,c,d], M. Rebagliato[b,e], A.F. Hernández[f]

" *Prenatal and to a lesser extent postnatal exposure to OPs may contribute to neurodevelopmental and behavioral deficits in preschool and school children.*"

The overall take home message

….. in terms of regulating chemicals we occasionally make mistakes …..

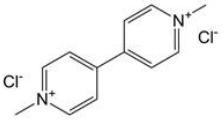


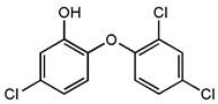
Time Magzine (June 30, 1947)

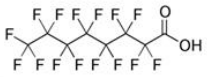
The great expectations held for DDT have been realized. During 1946, exhaustive scientific tests have shown that, when properly used, DDT kills a host of destructive insect pests, and is a benefactor of all humanity.

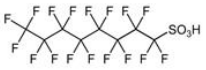
The overall take home message

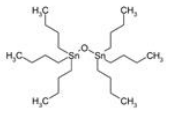
….. in terms of regulating chemicals we occasionally make mistakes ….. **repeatedly**









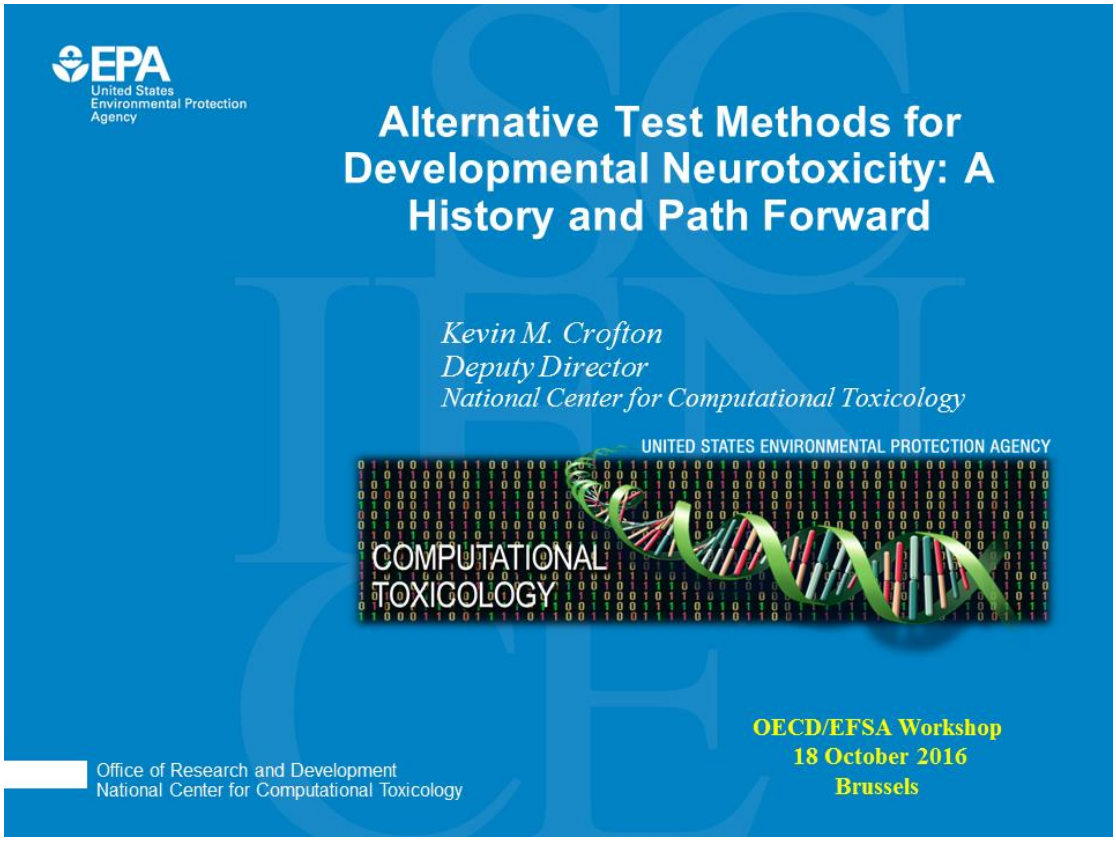


Conclusions

- There is sufficent evidence to suggest that early life exposures to certain chemicals (including pecticides) may have long term adverse health consequences for the developing offspring.
- Quality of existing studies equally reflects the complexity of capturing these effects
- A multidisciplinary approaches is needed and in vitro screening for neurotoxicity is a logical step in that direction

[PPT 2] Alternative Test Methods for Developmental Neurotoxicity: A History and Path Forward

Kevin Crofton, U.S. Environmental Protection Agency (USA)



Outline

- **Brief History of DNT Guidelines and Efforts to Promote In Vitro**
- **The Problems**
  - Evidence of Increasing developmental neuro 'diseases'
  - Thousands and thousands of chemicals with no hazard info
- **The Importance of Matching Data Type to the Decision Context – "fit for purpose"**
- **Demonstrating Progress**
- **Suggestions for Path Forward**

A Brief History of DNT
Historical Contributions to DNT Guidelines

Table 1. Historical contributions to the DNT guideline.

Date	Event	Summary	References
1960s–1980s	Published research on DNT and behavioral testing	Evidence that developmental exposure to chemicals and drugs can alter behavioral function in young and adult animals	Irwin 1968, Spyker and Smithberg 1972, Barlow and Sullivan 1975, Butcher et al. 1979, Butcher and Vorhees 1979, Vorhees et al. 1979, Butcher and Nelson 1985, Adams 1986
1978–1984	CBTS	Study to examine intra- and interlaboratory reliability and sensitivity of behavioral test methods	Buelke-Sam et al. 1985, Kimmel and Buelke-Sam 1985, Kimmel et al. 1985
1984			
1982–1985			
1985–1988			
1989			
1993–1997			
1995	IPCS	Interlaboratory study using neurotoxic chemicals to evaluate test validity, reliability, and measurement variability	Catalano et al. 1997, MacPhail et al. 1997, Tilson et al. 1997
2000	ILSI workshop on DNT testing	Workshop to review U.S. EPA DNT behavioral test methods, pharmacokinetics, and neuropathology	Cory-Slechta et al. 2001, Dorman et al. 2001, Garman et al. 2001, Mileson and Ferenc 2001
2003	Japanese Interlaboratory Study	Interlaboratory study using neurotoxic chemicals to determine sensitivity of behavioral measures	Okazaki et al. 2003
2003	Behavioral Test Methods Workshop	Expert workshop to address design, conduct, and analysis of behavioral tests for neurotoxicity evaluation	Slikker et al. 2005
2004–2008	ILSI RSI Working Group	Working group focused on variability, statistical analyses, positive controls, identification and analyses, interpretation of treatment-related effects, and application of DNT testing to public health protection	Fenner-Crisp et al. 2005, Crofton et al. 2008, Holson et al. 2008 Raffaele et al. 2008,Tyl et al. 2008

This work led to, and supported the development of EPA and OECD Guidelines

- EPA - 1991 (revised in 1998)
- OECD 2007

A Brief History of DNT
Efforts to Encourage In Vitro

A long-series of workshops have been held specifically to promote the development and use of in vitro DNT for replacement of animal testing and regulatory use.

- 2005 - In Vitro Alternative Methods for DNT, Ispra, Italy (Coecke at al. EHP, 2007)
- 2006 - DNT TestSmart I (Lein et al. EHP, 2007)
- 2008 - DNT TestSmart DNT II (Crofton et al. ALTEX 2011)
- 2011 - DNT TestSmart III (Bal-Price et al. ALTEX 2012)
- 2014 - DNT TestSmart IV
- 2014 - ISTNET DNT (Bal-Price et al., Arch Toxicol 2015)

- 2016 – OECD/EFSA Workshop

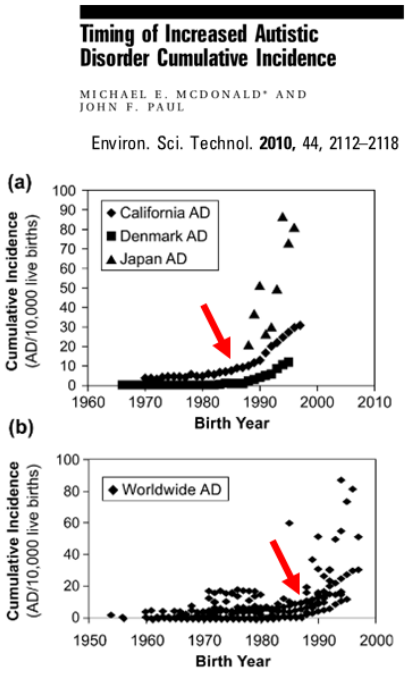
Alan Goldberg, 2006

Office of Research and Development

Problem: Evidence for Increasing Incidence of Neurodevelopmental Disorders

- Prevalence of neurodevelopmental diseases in children increased (Atladottir et al. 2015; Landrigan et al 2012)
- Overall estimates that 10-15% in children (Grandjean & Landrigan, Lancet 2014)
- Genetic factors account for no more than 30–40% (NRC, 2000)
- Includes: autism spectrum, ADHD, dyslexia, OCD, Tourette's

- McDonald and Paul (2010)
  - Identifies 'break point" for increases in autism
  - Provides a time frame for before and after



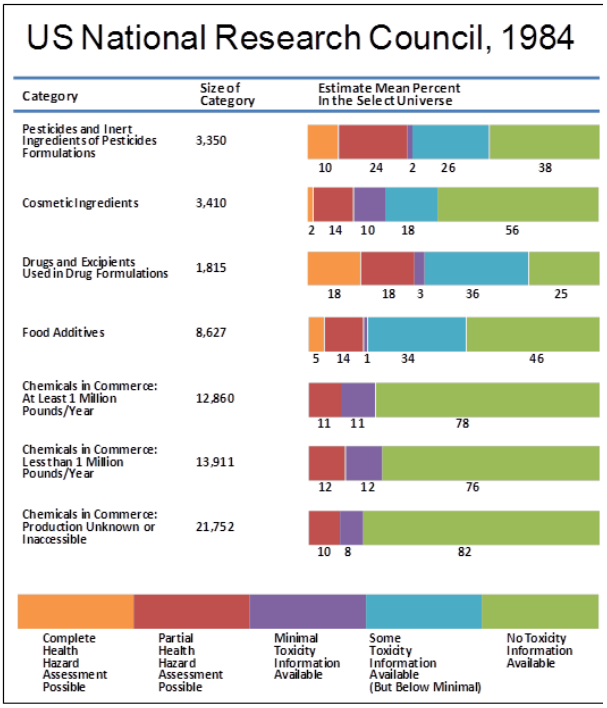
Problem: The Chemical Universe

1974 US NRC report

- Major challenge is too many chemicals and not enough data

- Estimated number of chemicals = 65,725

- Number of chemical with no toxicity data of any kind = 46,000



Matching Data Type and Uncertainties to Decision Context

It is critical to understand the uncertainties in the data

and

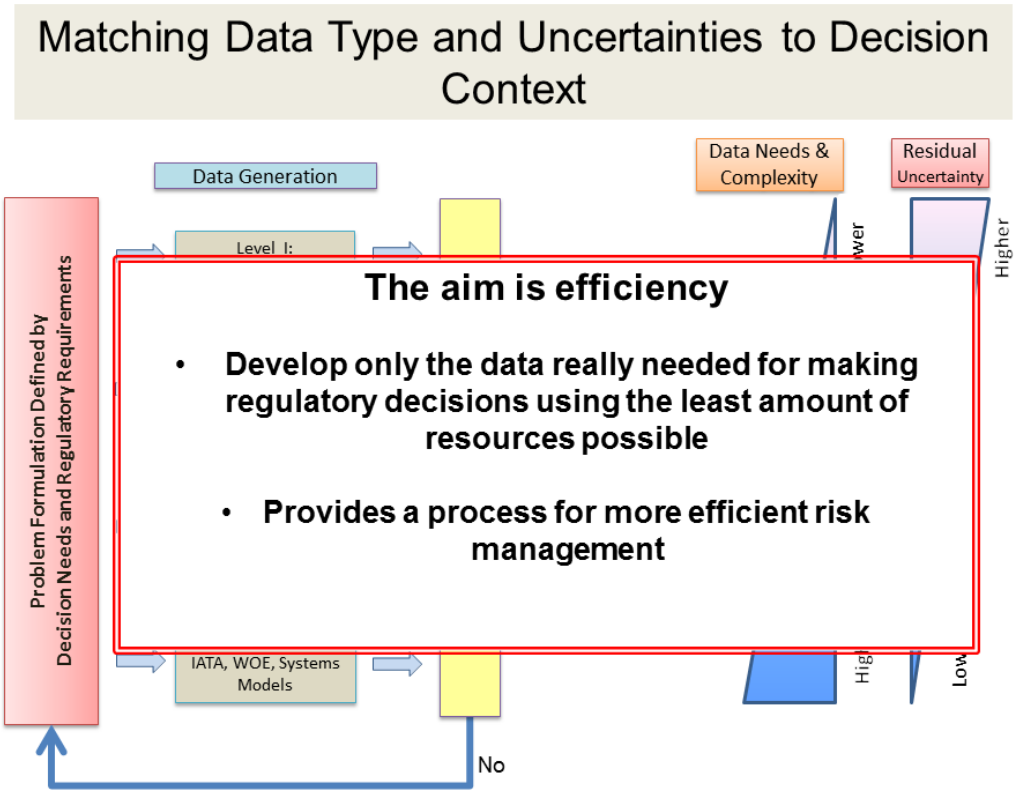
Match them to the regulatory decision context

Data Types & Chemical Risk Decisions

EPA Office	Assessment "Workflows"	Historical Throughput	Data Types
OPPTS	Premanufacture Notice (PMN) New chemicals Significant New Use Rule (SNUR) Existing chemicals	~1000/yr 90d/chem ~84,000 total	III (II)
	Current Chemical Risk *(new program)*	~10 total	I
	DFE / Green Chemistry	~2500	I, II, III
OSCP	Endocrine Screening Program	~10-20/year	
OPP	Pesticide registration (PR)	~10 new/yr ~50 old/yr	I
	Pesticide re-registration	~1000/yr 24,576 total	I
OW	Chemical Contaminant List	6yr ~6,000 total	I,II,III
	Regulatory Actions on CCL	6yr 90 total	I
	Unregulated Contaminant Monitoring	30/5yr	I
	Drinking Water Health Advisories (MCLs)	~80 total	II, III
ORD NCEA	IRIS	~3/yr ~540 total	I
	PPRTV	400-500	II,III

I. Data rich – Extensive guideline studies
II. Data partial – Some acute in vivo and in vitro data, SAR and exposure modeling
III. Data minimal to none – only chemical structure, SAR and exposure modeling

Courtesy of I. Shah



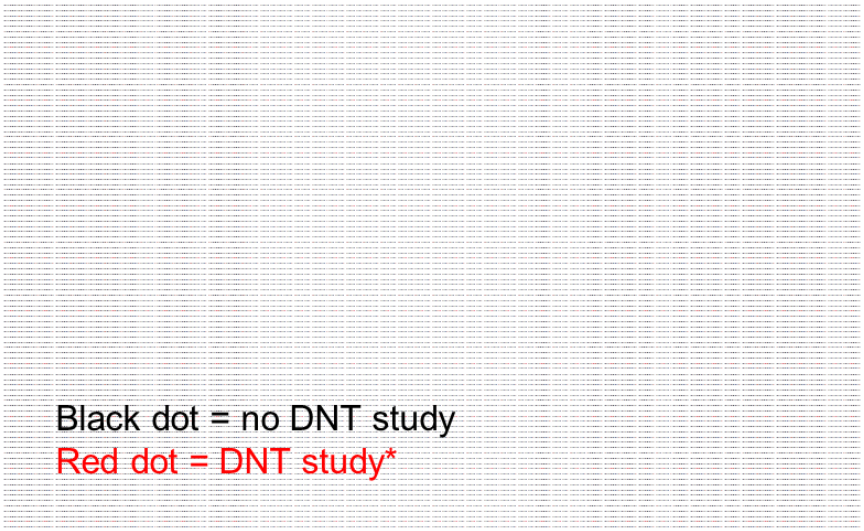
Progress To Date

In Vivo Guidelines

In Vitro Data

Progress to Date – In Vivo

How to visualize the problem of 60,000 Chemicals and not many DNT studies?



Office of Research and Development
National Center for Computational Toxicology

* ~0.2% DNT Guideline Studies

Progress to Date – In Vitro

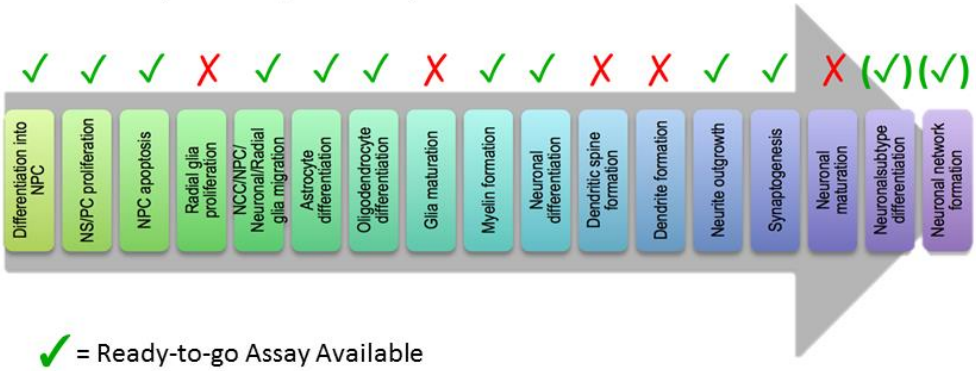
Critical Science Challenges for DNT*

- Develop and evaluate in vitro assays for application to DNT
- Develop reference chemicals for demonstration of predictability
- Generate data for lots of chemicals
- Develop tiered testing and decision frameworks
- Build open databases to share and compare methods and results

Based on DNT I, DNT 2, and 2007 Talk at CAAT 25th Anniversary Meeting

Progress to Date - Assays

- Over the past 2 decades there has been development of in vitro assays for a variety of DNT processes;



✓ = Ready-to-go Assay Available

✗ = No Ready-to-go Assay Available, yet cell system available

Fritsche, 2016

No reason not to start *fit-for-purpose* use

Progress to Date
Need for Reference Chemicals

- Over the past 5 years multiple reviews of in vivo and in vitro data to generate lists of reference chemicals
- Kadereit et al Front. Biosci 2012.
  - Criteria for selection and use of "gold standards"
  - List of XX chemicals
- Mundy et al 2015
  - GRADN list
  - 100 chemicals with evidence of development neurotoxicity
- Aschner et al ALTEX 2106
  - ~100 compounds (including negative controls) to address specificity, adversity and use of alternative test systems.
  - ~50 endpoint-specific controls and 33 "bona fide DNT toxicants"

Need consensus on lists

Progress to Date - Data Generation Examples

*

- There has been less progress on the generation of data for chemicals (see Fritsche EFSA/OECD Report)

- Data collections
  - Mundy & colleagues – synaptogenesis, proliferation, apoptosis, neurite outgrowth, viability
  - Leist & colleagues – neurite outgrowth, migration, viability
  - Shafer & colleagues – MEAs, viability
  - Biel et al (2015) - Proliferation, viability, neurite outgrowth, MEAs
  - NTP 80
    - Multiple labs and assays
  - EPA Organophosphates Project

* Based on my previous expectations and lack of patience!

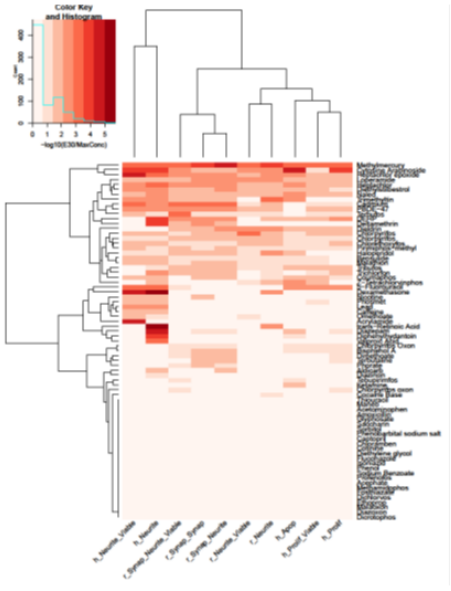
Mundy and colleagues Hierarchical Clustering of Potency of Multiple DNT Endpoints

Total of 70 chemicals

10 Endpoints
- human and rat cells
- viability
- neurite outgrowth
- synaptogenesis
- proliferation
- apoptosis

Values are - log(E_{30})
- Pink (0) = no effect
- darker red = more potent

- Ranking by clustering - combination of potency, neuro-endpoints and viability

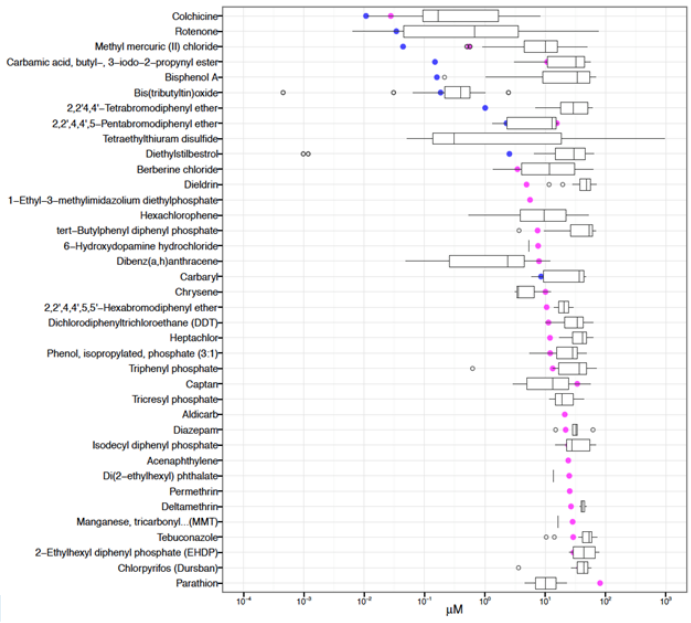


Allows prioritization by:
1. Overall potency
2. Selectivity for neurodevelopment endpoints (not shown)

Note: High priority chemicals tend to be similar for both approaches.

Courtesy of W. Mundy

Leist and colleagues – Neurite Outgrowth Comparison to Tox21 Assays



- Many compounds more sensitive in neurite outgrowth assays compared with current Tox21 assays
- *This suggests value of adding these models to expand current biological space of Tox21*
- May want to consider testing some of these compounds in vivo for further hazard characterization

iPSC
LUHMES
Tox21

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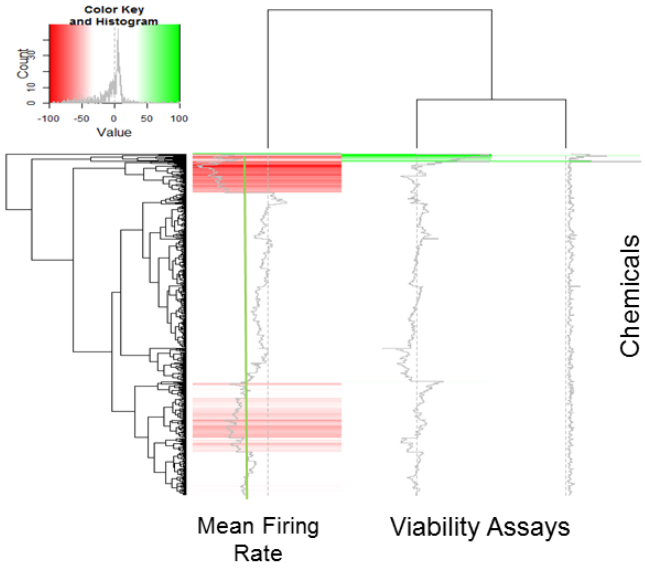
Courtesy of M. Leist

Shafer and colleagues Screening ToxCast Chemicals with MEAs

"Acute" Assay

- **1080 ToxCast** Phase 1& 2 single concentration
- 384 'hits'were then run in conc-response
- Good separation between cell viability and reduced firing rates
- Provides functional measure of neural activity



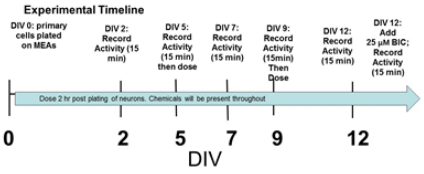
Office of Research and Development
National Center for Computational Toxicology

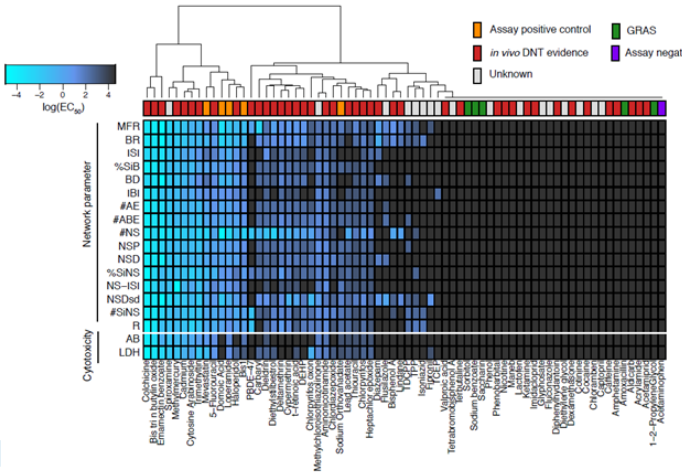
Public release of data release via ToxCastDB in 2017

Courtesy of T. Shafer

Shafer and colleagues
Results for "Developmental" MEA

- Total of 170 chemicals (so far)
  - Mundy List (70), NTP80 (50), ToxCast (50)
- 15 measures of neural activity
- Exposure throughout network development





- Allows prioritization by overall potency
- Provides functional measure of neural activity in a "developmental" context
- Can a signature pattern be developed that predicts targets?

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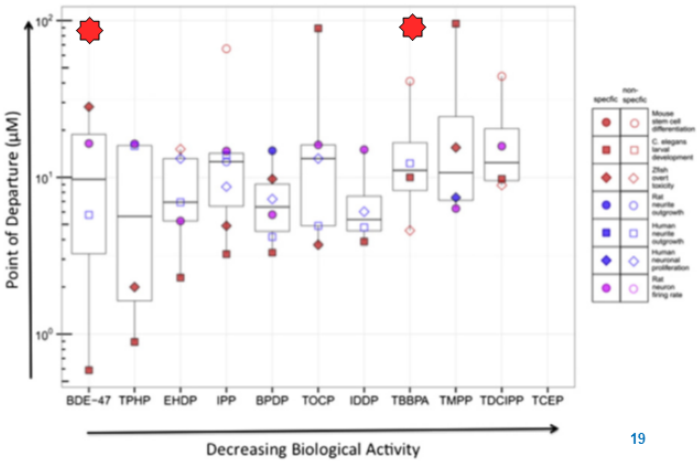
Courtesy of T. Shafer

Behl et al (2015) – NTP OP Flame Retardant Case Study

- Purpose: Compare BFRs to replacement OP-FRs via bioactivity
- Use battery approach – in vitro devtox and DNT assays
  - proliferation, viability, neurite outgrowth, MEAs, cytotoxicity, devtox assays
- 11 organophosphate and brominated flame retardants
- Compare in vitro PODs

Conclusions:
- *no one endpoint was always best*
- *similarity of bioactivity for replacements suggests need for follow-up testing*



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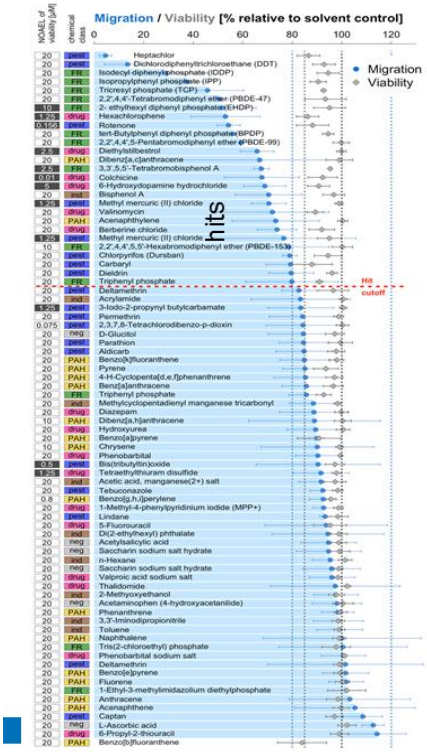
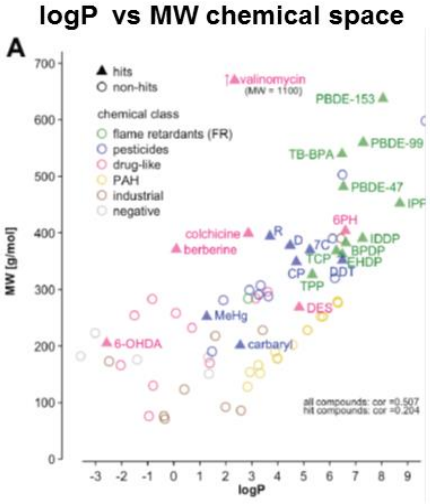


(Nyffeler et al., unpublished)

Leist and colleagues
NTP80 and Neural crest assay

- Measures both migration and viability with good separation for hits
- *Great example of how larger datasets allows for examination of relationships between chemical properties and bioactivity*

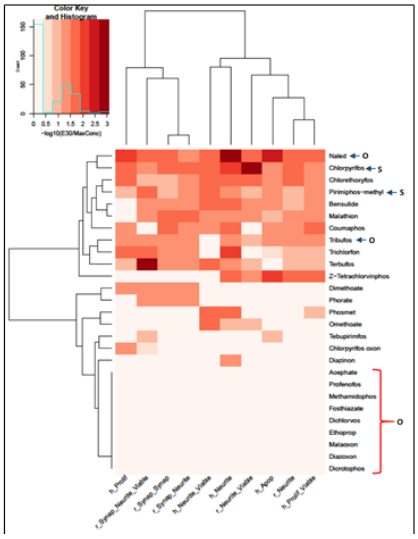


EPA OPP-NHEERL Organophosphate Project

- The effects of OPs on neurodevelopment are likely multi-target based. Some caused by AChE inhibition and others due to unknown mechanism(s).
  - Epi studies in children show DNT outcomes at doses that cause AChE inhibition.
- In 2015 OPP - ORD started a project to develop data for 27 OPs using in vitro DNT assays as well as zebrafish in order to:
  - *determine whether such data may be useful in reading across from data rich to data poor (no in vivo DNT data) chemicals*
- Work is ongoing

Preliminary Data

- In vitro only
- Suggest not all are the same
- Does not contain MEAs or zebrafish endpoints



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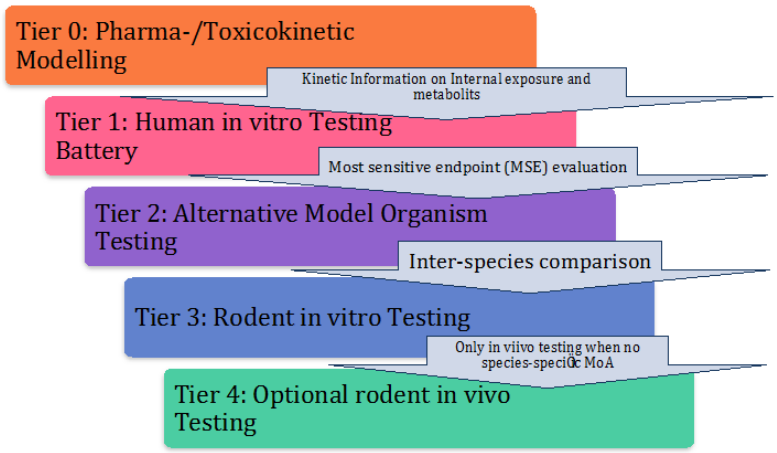
Courtesy of W. Mundy

Progress to Date
Tiered Testing & Decision Frameworks

Proposed tiered DNT testing :



Fritsche, 2016

Major Discussion Topic At Meeting

Progress to Date – Build Open Databases

Multiple databases available to deposit datasets
No use yet for DNT data

Progress to Date – In Vitro

Critical Science Challenges for DNT*

- ✓ Develop and evaluate in vitro assays for application to DNT
- ✓ Develop reference chemicals for demonstration of predictability
- Generate data for lots of chemicals
- Develop tiered testing and decision frameworks
- ✓ Build open databases to share and compare methods and results

*Based on DNT I, DNT 2, and 2007 Talk at CAAT 25th Anniversary Meeting

Ideas for Focusing Research Efforts Going Forward

- **Must develop data for MORE CHEMICALS – testing of large chemical libraries inform:**
  - Potential assay confounds - auto fluorescence, protein denaturation etc
  - Allows for better predictive models – read across,
  - Will foster development of DNT 'chemotypes"
  - Patterns across multiple assays at relevant concentrations will increase confidence in use for more than prioritization "risk" decisions
- **Better relationships between risk managers and scientists**
  - Don't just develop a new assay – develop assays and data that provide the information needed to make risk decisions
  - Scientists - talk to the risk managers here at the meeting – If you don't understand their problems how do you solve them?
- **Build data sharing opportunities**
  - Start combining work to compare across multiple labs and multiple types of assays

A Couple of Cautionary Issues

- **On the issue of "validation"**
  - Remember that the idea is "fit-for-purpose'
  - Amount of effort to validate for replacement of animal guidelines must be very different than use for prioritization or support for read across

- **Time is against us – technology is evolving at a very rapid pace**
  - New biotechnologies promise better biological coverage
  - Currently testing new 'global' genomics technologies that promise ability to tests entire genome for low prices
    - e.g., Biospyder – whole human genome on cell lysates
      http://biospyder.com/technology/
  - Don't wait for perfection
  - Always be willing to adapt to new and better technologies

  (remember - the DNT guidelines are based on technologies from the 70's and 80's)

"Do not let the perfect be the enemy of the good" *Voltaire*

"Do not let the perfect get in the way of developing and using in vitro data for use in risk assessments" *Crofton*

Office of Research and Development
National Center for Computational Toxicology

[PPT 3] EU regulatory perspective with special focus on pesticides
Susanne Hougaard Bennekou, Danish EPA (DNK) and Roland Solecki, German Federal Institute for Risk Assessment (DEU)

OECD/EFSA Workshop on DNT: the Use of Non-Animal Test Methods for Regulatory Purposes, Bruxelles 18-19 October 2016

Developmental Neurotoxicity

EU regulatory perspective with special focus on pesticides

Susanne Hougaard Bennekou

Andrea Gall and Roland Solecki

Regulatory DNT Approaches for Pesticides

Outline

- **What can be learned from regulatory authorities**
- **regarding DNT data requirements and testing strategies**
- **including alternative DNT assays?**

- **DIVING** into examples of
- how regulators from different countries handle ***In-vivo*** and ***In-vitro*** DNT data requirements?
- what is, or could be, the role of **alternative DNT assays** and **testing strategies** in their decision making?

- **Expectations:**
- describe specific approaches applicable for **DNT data requirements providing special emphasis on alternative DNT assays and testing strategies.**
- provide relevant cases in their area, and how they are usually handled.

Miljøministeriet
Miljøstyrelsen

Organophosphate metabolites in urine samples from Danish children and women

Measured in the Danish DEMOCOPHES population

Thit Aarøe Mørck[1]
Helle Raun Andersen[2]
Lisbeth E. Knudsen[1]

[1]University of Copenhagen
[2]University of Southern Denmark

145 women and 144 Children - 2011
Detectable concentrationen of OP metabolites in 90%
More than 4 different metabolites in 30%

Concentration (nmol/L)		GM [95% CI]	Median (p95)	Spearman's ρ
DMAP	Children	57.7 [48.2-68.4]	59.5 (318)	0.121
	Mothers	45.5 [37.9-54.3]	50.7 (245)	
DEAP	Children	35.9 [30.8-41.7]	37.8 (150	0.107
	Mothers	29.6 [25.1-34.5]	29.8 (135)	
DAP	Children	**111 [96.7-126]***	106 (387)	0.086
	Mothers	**84.8 [72.7-98.2]**	92.3 (386)	
Creatinine corrected (nmol/g creatinine)				
DMAP	Children	**60.4 [49.5-72.5]***	63.5 (378)	**0.228****
	Mothers	**47.3 [39.8-55.5]**	48.2 (251)	
DEAP	Children	37.5 [32.4-43.6]	39.3 (151)	0.091
	Mothers	30.8 [26.9-35.2	31.6 (122)	
DAP	Children	**116 [99.8-133]****	106 (515)	**0.203***
	Mothers	**88.1 [77.6-100]**	81.9 (286)	

Geometric means (gm) with 95% confidence intervals (ci), medians and 95 percentiles of the summed metabolites dmap, deap and dap in children (n=144) and mothers (n=145). The correlation coefficient spearman's rho is shown for correlations between mothers and children. Values below lod were set to lod/√2, dap was calculated as the sum of deap (sum of diethyl alkylphosphates): dep+ detp and dmap (sum of dimethyl alkylphosphates): dmp+dmtp. Significant differences between mothers and children by paired t-test and significant correlations measured by spearman's rho are marked in bold. * Significance level p<0.05, ** significance level p<0.01.

Miljøministeriet
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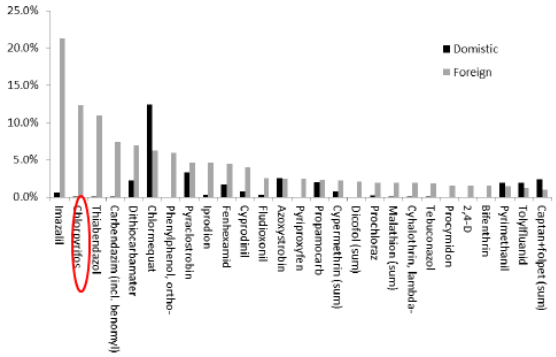
Context
Levels comparable to the rest of Europe
BUT higher than in the US (CDC data)

Source?
Very few OP's authorised for pesticide and biocide use in DK
Most likely source: Food not produced in DK

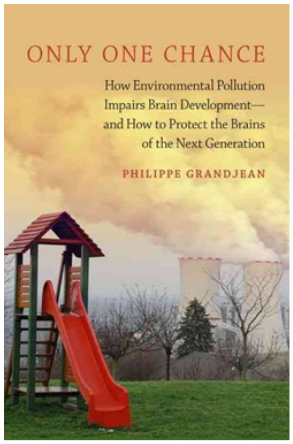
DTU

Pesticide Residues
Results from the period 2004-2011

DTU Fødevareinstituttet



Hazard Quotient below 1%
The fact that OP metabolites are found in the urine is not equal to an unacceptable risk
Status 2015
Still the most frequently found OP
The ADI was lowered and consequently MRL's
The exposure is expected to decline
Cumulative Risk?
The total exposure of OP's does still not pose an unacceptable risk
Case Closed?



"In longitudinal birth cohort studies based on the CHAMACOS-cohort and residents of New York City, maternal exposure to chlorpyrifos and other organophosphate insecticides in pregnancy was associated with neurobehavioural deficits in the children at least through 7 years of age (Bouchard et al. 2011; Eskenazi et al. 2007; Marks et al. 2010; Rauh et al. 2011; Rauh et al. 2006)."

The levels in DK/EU are above the "adverse effects levels in the US".
"Thus, studies of potential adverse health effects related to organophosphate exposure in European populations are needed".

MEDIA

Forskere slår alarm: Høj mængde pesticider hos danske børn

Hjalte Kragesteen | 18. maj 2016 kl. 7:10 | 9 kommentarer

Print

Landbrugets tunge ansvar: Masser af sprøjtemidler i danske børn

Udgivet maj 18, 2016 | Af Kjeld Hansen

NY RAPPORT: Koncentrationen af pesticid-rester i urinen hos danske børn er alarmerende højt, mener forskere på baggrund af en ny undersøgelse. De frygter blandt andet, at flere vil udvikle ADHD. Men Miljøstyrelsen ser ingen grund til panik. Det skriver Altinget.dk 18. maj

Professor Philippe Grandjean ser med stor bekymring på de høje niveauer af sprøjtegift-rester i danske børn urin.

Danska barn har "alarmerande" höga halter bekämpningsmedel

abc nyheter

Debatt: «Var årsaken til bankkrisen»

Dødstallene i Middelhavet stiger

SISTE NYHETER PENGER LIVET MOTOR REISE DATALIV VIDEO VÆRET A - Å

Bolig og interiør Familien Mat og drikke Mote Trening Helse Tester

Sprøytemidler funnet i barneurin

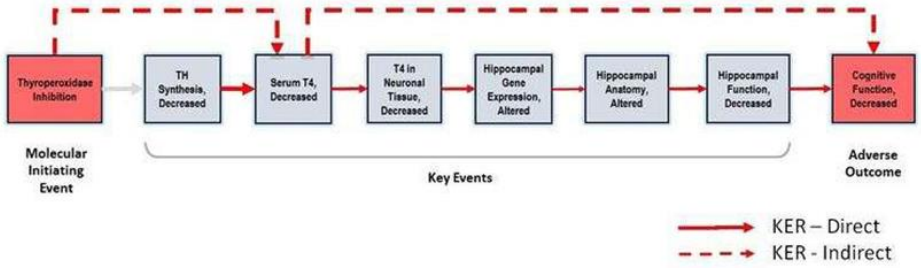
Oral Hearing of Minister in Parliament – June and September 2016

It is important that we as a society protect the coming generation against damaging substances. This is done by research, risk assessment and establishment of safe reference values

OECD/EFSA Workshop on DNT: the Use of No-Animal Test Methods for

Under Review

AOP42: XENOBIOTIC INDUCED INHIBITION OF THYROPEROXIDASE AND SUBSEQUENT ADVERSE NEURODEVELOPMENTAL OUTCOMES IN MAMMALS (INHIBITION OF THYROPEROXIDASE AND SUBSEQUENT ADVERSE NEURODEVELOPMENTAL OUTCOMES IN MAMMALS)



OECD/EFSA Workshop on DNT: the Use of No-Animal Test Methods for

Under development

- **AOP134: SODIUM IODIDE SYMPORTER (NIS) INHIBITION AND SUBSEQUENT ADVERSE NEURODEVELOPMENTAL OUTCOMES IN MAMMALS**
- **AOP54: INHIBITION OF NA+/I- SYMPORTER (NIS) DECREASES TH SYNTHESIS LEADING TO LEARNING AND MEMORY DEFICITS IN CHILDREN**
- **AOP8: UPREGULATION OF THYROID HORMONE CATABOLISM VIA ACTIVATION OF HEPATIC NUCLEAR RECEPTORS, AND SUBSEQUENT ADVERSE NEURODEVELOPMENTAL OUTCOMES IN MAMMALS**
- **AOP152: INTERFERENCE WITH THYROID SERUM BINDING PROTEIN TRANSTHYRETIN AND SUBSEQUENT ADVERSE HUMAN NEURODEVELOPMENTAL TOXICITY**

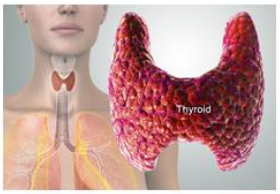
OECD/EFSA Workshop on DNT: the Use of No-Animal Test Methods for

European Food Safety Authority

Grouping of Pesticides for Cumulative Risk Assessment

287 chemical active substances were screened

Nervous system
(65 substances)



Thyroid system
(101 substances)

European Food Safety Authority

Effects on the thyroid system

Thyroid system

Chronic effects

22 substances:
Effects on the parafollicular (C-) cells or the calcitonin system

96 substances:
Substances affecting follicular cells and/or thyroid hormone (T3/T4) system

European Food Safety Authority EFSA Journal 2013;11(12):3471

Scientific Opinion on the developmental neurotoxicity potential of acetamiprid and imidacloprid[1]

EFSA Panel on Plant Protection Products and their Residues (PPR)[2, 3]
European Food Safety Authority (EFSA), Parma, Italy

The PPR Panel encourages the definition of clear and consistent criteria at EU level to **trigger** submission of mandatory DNT studies, which could include development of an **integrated DNT testing** strategy composed of robust, reliable and **validated *in vitro* assays and other alternative methods complementary to the *in vivo* TG 426 for assessing the DNT potential of substances.**

Thank you for your attention!

Welcome

Dr. Roland Solecki, BfR

FEDERAL INSTITUTE FOR RISK ASSESSMENT

BfR
Bundesinstitut für Risikobewertung

Developmental Neurotoxicity

EU regulatory perspective with special focus on pesticides

Andrea Gall and Roland Solecki
German Federal Institute for Risk Assessment, Berlin

Regulatory DNT Approaches for Pesticides
Legal framework

Plant Protection Products

Regulation (EC) 1107/2009

Data requirements legally binding (Reg. (EU) 283/2013)

- ***5.6.2. Developmental toxicity studies***
- Developmental toxicity studies with other relevant data and information on the active substance, shall be sufficient to permit the assessment of effects on embryonic and foetal development, following repeated exposure to the active substance…
- When indicated by observations in other studies or the mode of action of the test substance, supplementary studies or information may be required to provide information on the postnatal manifestation of effects such as **developmental neurotoxicity**.
- **5.7.1. *Neurotoxicity studies in rodents***
- Neurotoxicity studies in rodents shall provide sufficient data to evaluate the potential neurotoxicity of the active substance (neurobehavioural and neuropathological effects) after single and repeated exposure.

Regulatory DNT Approaches for Pesticides
Legal framework

Biocidal Products in Europe

Regulation (EC) 528/2012

Data requirements legally binding (A.-II Reg. (EU) 528/2012)

- ***Article 19 Conditions for granting an authorisation***
  …the simplified authorisation procedure acc. Article 25
  A biocidal product shall not be authorised where:…
  it has **developmental neurotoxic** or immunotoxic **effects**.

ANNEX II INFORMATION REQUIREMENTS FOR AS

- **8.10.1. Pre-natal developmental toxicity study** (Core Data Set)
- **8.10.3. Further pre-natal developmental toxicity study** (Additional Data Set)
- **8.13.2. Neurotoxicity including DNT** (Additional Data Set)
  if there is any evidence knowledge of the mechanism of action or from repeat dose studies that the a.s. may have neurotoxic or
  developmental neurotoxic properties
  then additional information or specific studies will be required.

Regulatory DNT Approaches for Pesticides
Legal framework

Regulation (EC) No. 1107/2009 (PPP):

„An active substance,…, shall only be approved [...]

- if it is not or has not to be classified, in accordance with the provisions of Regulation (EC) No 1272/2008, as toxic for **reproduction category 1A or 1B** [...]“

Regulation (EU) No. 528/2012 (BP):

“ the following active substances shall not be approved: […]

- active substances which have been classified in accordance with Regulation (EC) No 1272/2008 as, or which meet the criteria to be classified
  as toxic for **reproduction category 1A or 1B**

unless…negligible exposure/risk

Regulatory DNT Approaches for Pesticides
Case studies, how DNT data usually handled

Risk Assessment and Setting of Reference Values; Acute Reference Dose (ARfD) and Acceptable Daily Intake (ADI)

- **Data availability**:
  - Developmental toxicity studies
  - Acute and repeated neurotoxicity
  - Developmental neurotoxicity study (not mandatory in Europe)
- **Relevant effects:**
  - Embryo-/fetotoxicity (e.g. death, growth retardation)
  - Structural defects (malformations, variations)
  - Neurological effects (structural, neurobiochemical, behaviour)
- **Conclusions**:
  - Developmental, multigeneration, acute and short-term neurotoxicity studies, but also developmental (neurotoxic) effects relevant for ARfD and ADI.
  - Consider length of the critical window, kinetics, mechanism.
  - Indications from in vitro DNT Testing considered to be supportive

Regulatory DNT Approaches for Pesticides
Case studies, how DNT data usually handled

2002 - Report of the Joint Meeting of the
FAO Panel of Experts on Pesticide Residues

Results of DNT studies summarized in a EPA working paper were reviewed to examine the impact of DNT studies on ARfD and ADI:

- DNT studies on 14 pesticides evaluated by the US EPA were reviewed.
- Both generic and chemical-specific experimental DNT study designs were considered.
- Toxicity end-points of each DNT study and four related studies compared (developmental, multigeneration, acute/short-term neurotoxicity studies)
- The comparison showed that, in general, the majority of DNT studies did not identify significantly lower NOAELs and LOAELs compared to those of the other four related studies.
- With OP pesticides, functional and pathological effects in DNT studies not at lower doses than those at which cholinesterase inhibition was observed.

Regulatory DNT Approaches for Pesticides
Case studies, how DNT data usually handled

Critical Reviews in Toxicology, 2010; 40(1): 24–34

informa healthcare

REVIEW ARTICLE

A retrospective analysis of Acute Reference Doses for pesticides evaluated in the European Union

Roland Solecki, Tomas Moeller, Michael Herrmann, and Bernd Stein

Department of Chemical Safety, Federal Institute for Risk Assessment, Berlin, Germany

Table 1. Pesticides grouped by study type used for ARfD derivation in

Studies used for ARfD derivation	Number of substances	Percentage (%)
ARfD based on special studies	8	4.0
ARfD based on acute neurotoxicity studies in rats	20	10.1
ARfD based on repeated-dose studies in rats or dogs	16	8.1
ARfD based on multi-generation reproduction studies in rats	3	1.5
ARfD based on developmental toxicity studies in rats or rabbits	53	26.8
ARfD based on DNT studies in rats	2	1.0
ARfD based on human data	1	0.5
No ARfD was considered necessary	95	48.0

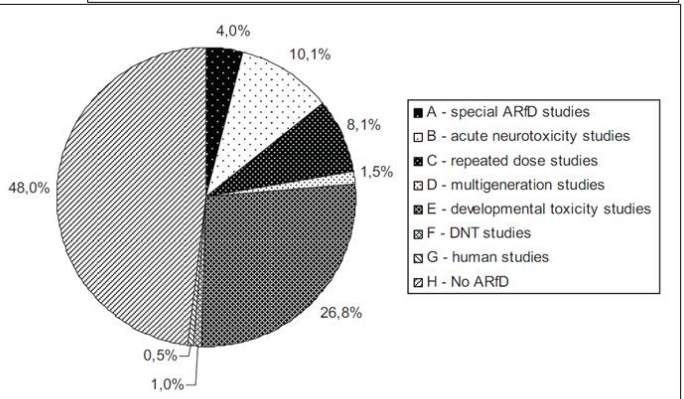


Figure 1. ARfD derivation in the EU pesticide evaluation program.

Regulatory DNT Approaches for Pesticides
Case studies, how DNT data usually handled

HSE

Supporting Publications 2013:EN-413

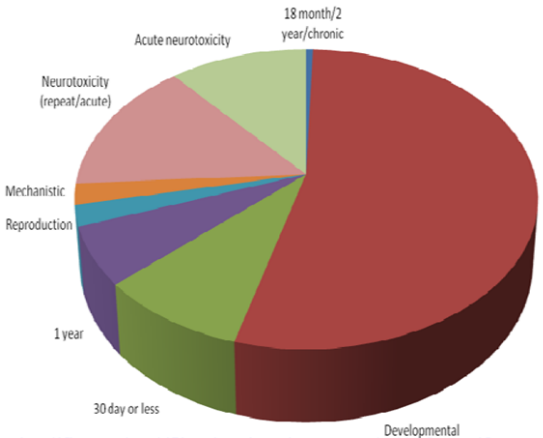
EXTERNAL SCIENTIFIC REPORT

Investigation of the state of the art on identification of appropriate reference points for the derivation of health-based guidance values (ADI, AOEL and AAOEL) for pesticides and on the derivation of uncertainty factors to be used in human risk assessment[1]

Chemicals Regulation Directorate, Health & Safety Executive, UK

Frequency of main study types used to set ARfD

18 month / 2 year / chronic	1%
1 year	8%
90 day	8%
2 - 30 day	10%
Mechanistic	3%
Developmental	58%
Reproduction	3%
Neurotoxicity (repeat /acute)	19%
Acute neurotoxicity	16%



http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/413e.pdf

Regulatory DNT Approaches for Pesticides
Evaluation of DNT studies on pesticides

The impact of DNT studies on ARfD and ADI setting:

- Available DNT studies on pesticidal active substances in plant protection products were analysed to examine.
- DNT studies on **35 substances** **out of 485 pesticidal active substances**, currently approved in the EU were reviewed:
  - **21 insecticides, 7 fungicides, 6 herbicides, 1 acaricide**
  - **19 positive tested substances** out of the 35 available screening and/or DNT studies were considered:
  - **15 insecticides, 2 herbicides & 2 fungicides**
  - 18 tested substances revealed evidence of DNT as well as neurotoxicity
  - 1 DNT positive tested fungicide did not reveal any evidence of neurotoxicity in adult rats (acute, FOB in 28-d, DNT)

Regulatory DNT Approaches for Pesticides
Evaluation of DNT studies on pesticides

The impact of DNT studies on ARfD and ADI setting:

- Reference values for the 19 positive tested pesticidal active substances are currently based on:
- **2 substances on DNT studies**
- 1 substance on a DNT study, but on developmental toxic effects
- 1 substance on an in-vivo Comparative Cholinesterase Assay
- 8 substances (ARfD) on developmental toxicity studies (rat, rabbit)
- 7 substances (ARfD) on neurotoxicity studies (acute, repeated)

- DNT is covered by both, ADI & ARfD in 15/19 studies
- DNT is covered by ADI, only in 2/19 studies (ARfD has to be re-evaluated)
- DNT not covered by ADI & ARfD in 1/19 studies (currently under discussion)
- 1 additional (positive) DNT has not yet been peer reviewed at EU level

Regulatory DNT Approaches for Pesticides
Case studies, how DNT data usually handled

Neonicotinoid insecticides under discussion:

Acetamiprid,

current EU ADI 0.07 mg/kg bw based on 2-yr & 2-generation, rat;
AOEL 0.124 mg/kg bw/d based on 90-d, rat;
ARfD 0.1 mg/kg bw based on acute neurotoxicity, rat.

PPR Panel (2013) considered that the current reference values may not be protective enough for possible DNT and ,
recommends a more conservative NOAEL of 2.5 mg/kg bw/d
[note: based on *supplementary DNT study, supported by in-vitro data*]
ADI, ARfD & AOEL**, which all should be set at **0.025 mg/kg bw/d
New and more reliable DNT data are required, the point of departure can be revised.

Imidacloprid,

current EU ADI 0.06 mg/kg bw based on 2-yr, rat;
AOEL & ARfD 0.08 mg/kg bw/d based on 28-d & 90-d, dog,
supported by subchronic neurotoxicity, rat.

PPR Panel (2013): *current ARfD and AOEL not be protective enough for potential DNT*
recommends to conservatively lower these reference values to the same level as ADI 0.06 mg/kg bw

Regulatory DNT Approaches for Pesticides
Case studies, how DNT data usually handled

Glufosinate-ammonium

ADI & ARfD: **0.021 mg/kg bw** based on the NOAEL of 6.3 mg/kg bw/d (developmental toxicity, rabbit) and application of a safety factor of 300.

ARfD is considered to be adequately protective for any reproductive and developmental neurotoxic effects:

Rat		**NOAEL**	**LOAEL**
Developmental toxicity study	Maternal toxicity	10 mg/kg bw/d	50 mg/kg bw/d
	Embryo/foetal toxicity	10 mg/kg bw/d	50 mg/kg bw/d
DNT study	Maternal toxicity	69 mg/kg bw/d	292 mg/kg bw/d
	DNT	14 mg/kg bw/d	69 mg/kg bw/d
Rabbit			
Developmental toxicity study	Maternal toxicity	6.3 mg/kg bw/d	20 mg/kg bw/d
	Embryo/foetal toxicity	6.3 mg/kg bw/d	20 mg/kg bw/d

Regulatory DNT Approaches for Pesticides
Case studies, how DNT data usually handled

Chlorpyrifos

EU (2005), ARfD of 0.1 mg/kg bw based on:

- Acute neurotoxicity study in rats, NOAEL of 10 mg/kg bw
- Single oral gavage dose, no inhibition of brain AChE at 10 mg/kg bw
- Scientifically/ethically valid study in volunteers, NOEL was 1 mg/kg bw

As a part of the re-registration for Chlorpyrifos, the USEPA called for an **Comparative Cholinesterase Assay (CCA) study in rats** to determine, if age-related sensitivities to ChE inhibition exist:

- NOAELs after acute/repeated exposure are the same in pups and adults
- No clear evidence, that pups are more sensitive

New available toxicological data lowered the reference value (2014):
ARfD of 0.005 mg/kg bw, based on acute CCA, rat

Regulatory DNT Approaches for Pesticides
Summary/Take Home Message

- Data requirements for the evaluation of plant protection products and biocidal products in the EU are legally binding.
- DNT testing is not mandatory, but may be required for pesticidal active substances, if indications for DNT effects from other mandatory studies.
- DNT studies on **35 out of 485 pesticidal AS** approved in EU are available, 19 revealed positive in vivo evidence of DNT, majority not tested for DNT.
- Reference values for 2 positive tested substances are currently based on DNT studies, 2 are currently under discussion.
- In positive tested substances, DNT effects are covered by risk assessment including both ADI and ARfD, or at least the ADI.
- DNT testing can be considered not sufficient, although the majority of risk assessments can be considered protective for positive in vivo DNT effects.
- For regulatory purposes, identification of DNT compounds by an adequate *in vitro* testing battery is considered essential as a first screening step.

[PPT 4] Developmental neurotoxicity under the REACH
Hannele Huuskonen, European Chemicals Agency (ECHA)

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Developmental neurotoxicity & REACH

Hannele Huuskonen

DNT WS 2016

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REACH Article 1

- The purpose of this Regulation is to ensure a high level of protection of human health and the environment, including the promotion of alternative methods for assessment of hazard of substances, as well as the free circulation of substances on the internal market while enhancing competitiveness and innovation.

Information requirements for developmental neurotoxicity

- **REACH**
  - Not a standard information requirement
  - Based on a particular concern within an extended one-generation reproductive toxicity study
  - Based on a particular concern under repeated dose toxicity?
- **C&L aspects**
  - Information must be applicable also for C&L

Reproductive toxicity information requirements under REACH (cumulative)

- At 10 – 100 tonnage band (Annex VIII)
  - OECD TG 421 or 422
  - EU TM B.31/OECD TG 414 or **B.56/OECD TG 443** may be proposed
- At 100 – 1000 tonnage band (Annex IX)
  - EU TM B.31/OECD TG 414 (1st species; + 2nd species if triggered)
  - EU TM **B.56/OECD TG 443** if triggered (results from e.g. 28- , 90-day or screening study or other concern)
- Over 1000 tonnage (Annex X)
  - EU TM B.31/OECD TG 414 (1st + 2nd species)
  - EU TM **B.56/OECD TG 443**

EOGRTS design?

- **Aspects specified for REACH:**
  - Length of the premating exposure duration
    - Starting point 10 weeks, can be shortened based on substance specific justifications
    - ECHA Guidance, reflecting Recital 7 of Regulation (EU) 2015/282
  - Highest dose level should be selected with the aim to produce some toxicity
    - ECHA Guidance, reflecting Recital 7 of Regulation (EU) 2015/282
  - Extension of Cohort 1B based on certain criteria (Column 2)
  - Inclusion of **DNT Cohorts 2A and 2B** based on triggers (Column 2)
  - Inclusion of Cohort 3 based on triggers (Column 2)

Relevant REACH processes

- **Dossier evaluation**: to fulfil data gaps for SIR and column 2 criteria for triggers
  - **Compliance check evaluation**: ECHA selects the registration dossier to be evaluated, requests a specific study design if missing
  - **Testing proposal evaluation**: The registrant proposes the study and the study design (following ECHA Guidance) if information not covered by an adaptation
- **Substance evaluation**: to request further data beyond the SIR based on further concern
  - MSCAs evaluates

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Inclusion of DNT Cohorts 2A and 2B in an EOGRTS

- Need to be included if there is a **particular** concern on (developmental) neurotoxicity justified by any of the following:
  - existing information on the **substance itself** derived from relevant available *in vivo* or non-animal approaches, or
  - **specific mechanism/modes of action** of the substance with an association to (developmental) neurotoxicity, or
  - existing information on effects caused by **substances structurally analogous** to the substance being studied, suggesting such effects or mechanisms/modes of action

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ECHA Guidance: DNT Cohorts, examples

- **Specific mechanisms/mode of action** that has been closely linked to (developmental) neurotoxicity effects
  - (Adult) brain cholinesterase inhibition (by 20%)
  - Relevant changes in thyroid hormone levels or signs of thyroid toxicity indicating such changes
  - Specific hormonal mechanisms/modes of action with clear association with the developing nervous system, such as oestrogenicity (Fryer *et al.*, 2012) and anti-androgenicity (Pallarés *et al.*, 2014)(organ weight changes/effects described in OECD GD 150 are used to identify these modes of actions)

Other options for DNT studies

- Column 2:
- *"Other studies on developmental neurotoxicity and/or developmental immunotoxicity instead of cohorts 2A/2B (developmental neurotoxicity) and/or cohort 3 (developmental immunotoxicity) of the Extended One-Generation Reproductive Toxicity Study may be proposed by the registrant in order to clarify the concern on developmental toxicity."*

- To be considered if there is no concern for reproductive toxicity, only for DNT, or if a separate study can be justified (e.g., more/further parameters needed)

Use of non-animal approaches - General adaptions REACH

- Annex XI, Sections…
  - 1.1 Use of existing data
  - 1.2 Weight of evidence
  - 1.3 (Q)SAR
  - 1.4 *In vitro* methods
  - 1.5 Grouping of substances and read-across approach
  - 2 Testing is technically not possible
  - 3 Substance-tailored exposure-driven testing
- Data must be adequate for classification and labelling and risk assessment

Classification of DNT effects

- Data should allow adequate evaluation and classification categorisation + risk assessment – also if non-animal approaches are part of data
  - High confidence level is needed for decision on categorisation
- Depending on the study design/test, the effects may reflect neurotoxicity or developmental neurotoxicity
  - Classification for neurotoxicity or for developmental toxicity?
  - Classification and categorisation are risk management measures
  - Important because of different regulatory down stream consequences in other legislations
- If developmental origin can be concluded then classification for developmental toxicity
- No experience yet

Classification categorisation for neurotoxicity

- Specific target organ toxicity (STOT)
- Single exposure:
  - **STOT-SE Category 1**
  - **STOT-SE Category 2**
- Repeated dose toxicity
  - **STOT-RE Category 1**
  - **STOT-RE Category 2**
  - **STOT-RE Category 3**
- No classification

STOT-SE/RE Category 1

- Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following single/repeated exposure

- Substances are classified in Category 1 for specific target organ toxicity (single/repeated exposure) on the basis of:

  (a) reliable and good quality evidence from human cases or epidemiological studies; or

  (b) observations from appropriate studies in experimental animals in which significant and/or severe toxic effects of relevance to human health were produced at generally low exposure concentrations. Guidance dose/concentration values are provided below … to be used as part of weight-of-evidence evaluation.

STOT-SE/RE Category 2

- Substances that, on the basis of evidence from studies in experimental animals can be presumed to have the potential to be harmful to human health following single/repeated exposure
  - Substances are classified in Category 2 for specific target organ toxicity (single/repeated exposure) on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations. Guidance dose/concentration values are provided below … in order to help in classification.

- In exceptional cases, human evidence can also be used to place a substance in Category 2.

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STOT RE 1/2

- Significant toxic effects in a 90-day repeated-dose study conducted in experimental animals are seen to occur at or below the guidance values:

Route	Species	Units	RE 1 Dose/ concentration	RE 2 Dose/ concentration
Oral	(rat)	mg/kg bw/day	C≤10	10<C≤100
Dermal	(rat or rabbit)	mg/kg bw/day	C≤20	20<C≤200
Inhalation (gas)	(rat)	ppmV/6h/day	C≤50	50<C≤250
Inhalation (vapour)	(rat)	mg/litre/6h/ day	C≤0.2	0.2<C≤1.0
Inhalation (dust/mist/ fume)	(rat)	mg/litre/6h/ day	C≤0.02	0.02<C≤0.2

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Classification categorisation, developmental toxicity

- Regarding to reproductive toxicity (including developmental toxicity) the categorisation is as follows:
  - **Repr 1A**: mainly based on information on humans
  - **Repr 1B**: mainly based on information on animal studies, findings considered relevant to humans, not secondary to other toxicity and severe and/or high incidence [= clear evidence]
  - **Repr 2**: findings that do not warrant categorisation to Repr 1B [=some evidence]
  - No classification
- Reproductive toxicity includes according to CLP:
  - adverse effects on **sexual function and fertility** in adult males and females
  - **developmental toxicity** in the offspring

Adverse effects on development of the offspring

- Developmental toxicity includes, in its widest sense, any effect which interferes with normal development of the conceptus, either before or after birth, and resulting from exposure of either parent prior to conception, or exposure of the developing offspring during prenatal development, or postnatally, to the time of sexual maturation. However, it is considered that classification under the heading of developmental toxicity is primarily intended to provide a hazard warning for pregnant women, and for men and women of reproductive capacity. Therefore, for pragmatic purposes of classification, developmental toxicity essentially means **adverse effects induced during pregnancy, or as a result of parental exposure**. These effects can be manifested at any point in the life span of the organism. The major manifestations of developmental toxicity include (1) death of the developing organism, (2) structural abnormality, (3) altered growth, and (4) functional deficiency.

Test methods in REACH

- Test methods shall be regularly reviewed and improved… (REACH Art 13(2))

- While the classification of any substance or mixture may be carried out on the basis of available information, the available information to be used for the purposes of this Regulation should preferably have been generated in accordance with the test methods referred to in Regulation (EC) No 1907/2006, transport provisions or international principles or procedures for the validation of information, so as to ensure quality and comparability of the results and consistency with other requirements at international or Community level. The same test methods, provisions, principles and procedures should be followed where the manufacturer, importer or downstream user chooses to generate new information. (CLP Recital 21)

Assessment and validation of alternative test methods

The test methods in Commission Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) are regularly reviewed and improved with a view to reducing testing on vertebrate animals and the number of animals involved. The European Centre for the Validation of Alternative Methods (ECVAM) of the Commission's Joint Research Centre plays an important role in the scientific assessment and validation of alternative test methods. (CLP Recital 26)

Non-animal approaches

- Information on intrinsic properties of substances may be generated by means other than tests, provided that the conditions set out in Annex XI are met. In particular for human toxicity, information shall be generated whenever possible by means other than vertebrate animal tests, through the use of alternative methods,... (REACH Art 13(1))
- The generation of information by alternative means offering equivalence to prescribed tests and test methods should also be allowed, ...(REACH Recital 38)
- Where new tests are carried out for the purposes of this Regulation, tests on animals within the meaning of Directive 86/609/EEC shall be undertaken only where no other alternatives, which provide adequate reliability and quality of data, are possible.(CLP Art 7)

GLP and other international standards

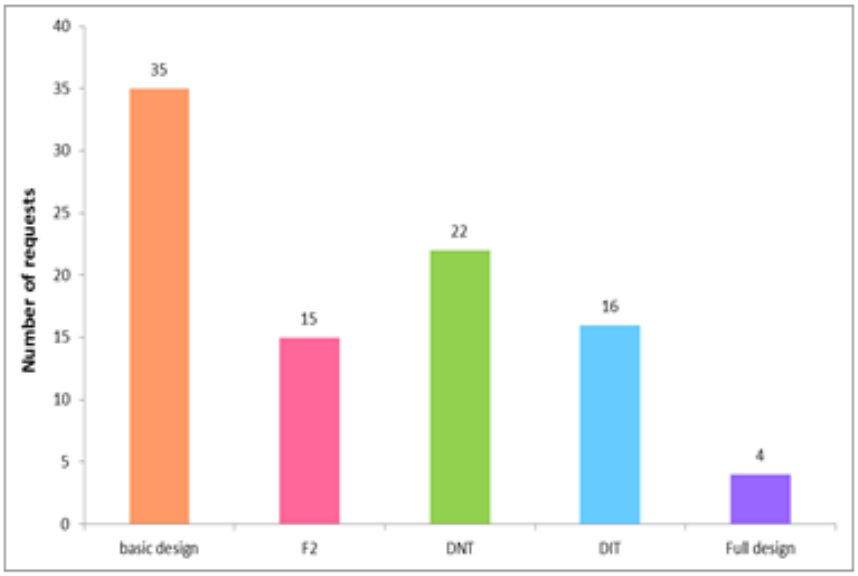
- … and toxicological tests and analysis shall be carried out in compliance with the principles of good laboratory practise… and other international standards recognised as being equivalent by Commission or the Agency… (REACH Art 13(4))
- Where the manufacturer, importer or downstream user carries out new ecotoxicological or toxicological tests and analyses, these shall be carried out in compliance with Article 13(4) of Regulation (EC) No 1907/2006. (CLP Art 8)

Regarding to biological availability…

Specific cases requiring further evaluation

- (b) conclusive scientific experimental data show that the substance or mixture is not biologically available and those data have been ascertained to be adequate and reliable (CLP Art 12)
- Testing … may be omitted where justified by information on exposure and implemented risk management measures as specified in Annex XI, section 3 (REACH Art 13(1))

Study expansions of EOGRTS in draft decisions
(distribution may change in final decisions)



Summary (1)

- Under REACH Regulation DNT can be required based on justified concern within an EOGRTS (under DEv and SEv)
- OECD TG 426 or other studies/tests/approaches may be required under Sev (concern-based)
- Registrants may provide OECD TG 426 or other DNT studies instead of EOGRTS DNT Cohorts
- Registrants may provide information from non-animal (alternative) approaches as a general adaptation according to Annex XI
  - Equivalent information

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Summary (2)

- Available data should allow risk assessment (DNEL derivation) and classification and labelling, including categorisation
- Available data should allows adequate evaluation of data and classification categorisation between categories 1, 2 and no classification
- Classification for neurotoxicity (STOT-SE/RE 1 or 2) or developmental toxicity (Repr 1B) have significantly different downstream consequences

[PPT 5] US EPA's Regulatory Perspective on Developmental Neurotoxicity Studies: A Focus on Pesticides

Elissa Reaves, U.S. Environmental Protection Agency (USA)

U.S. Regulatory Perspective on Developmental Neurotoxicity Studies: A Focus on Pesticides

October 18, 2016

Elissa Reaves, Ph.D
Acting Associate Director
Health Effects Division
Office of Pesticide Programs

Introduction

- USEPA's Office of Pesticide Programs is a licensing program regulating pesticide products in the U.S.
  - Review effects of pesticides on human and ecological health
  - OPP is data rich
    - Acute, subchronic, developmental, reproductive, chronic/cancer, dermal, inhalation
    - Flexibility to waive or require more data
    - DNT is a conditionally required study

Introduction

- 870.6300 Developmental Neurotoxicity; footnotes 27-29
- Footnote 27. An information-based approach to testing is preferred, which utilizes the best available knowledge on the chemical (hazard, pharmacokinetic, or mechanistic data) to determine whether a standard guideline study, an enhanced guideline study, or an alternative study should be conducted to assess potential hazard to the developing animal, or in some cases to support a waiver for such testing. Registrants should submit any alternative proposed testing protocols and supporting scientific rationale to the Agency prior to study initiation.

Introduction

- Footnote 28. Study required using a weight-of-evidence approach considering:
- (i) The pesticide causes treatment-related neurological effects in adult animal studies (*i.e.*, clinical signs of neurotoxicity, neuropathology, functional or behavioral effects).
- (ii) The pesticide causes treatment-related neurological effects in developing animals, following pre- and postnatal exposure (*i.e.*, nervous system malformations or neuropathy, brain weight changes in offspring, functional or behavioral changes in the offspring).

Introduction

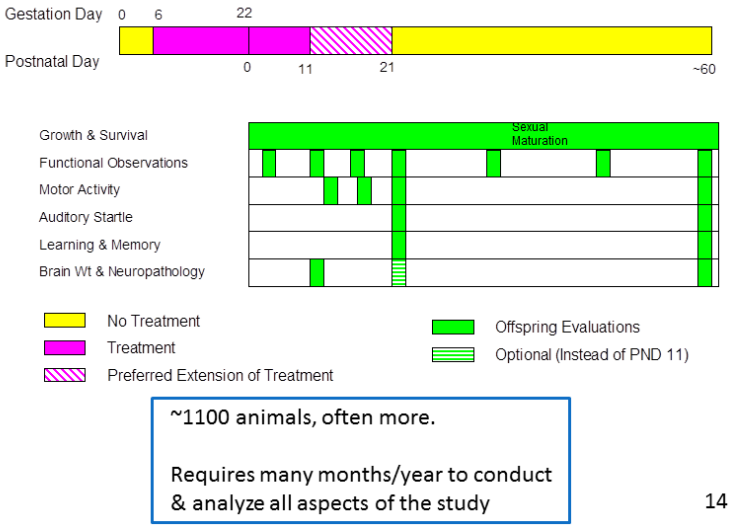
- Footnote 28. Study required using a weight-of-evidence approach considering:
- (iii) The pesticide elicits a causative association between exposures and adverse neurological effects in human epidemiological studies.
- (iv) The pesticide evokes a mechanism that is associated with adverse effects on the development of the nervous system (e.g., SAR relationship to known neurotoxicants, altered neuroreceptor or neurotransmitter responses).
- Footnote 29. The use of a combined study that utilizes the 2-generation reproduction study in rodents as a basic protocol for the addition of other endpoints or functional assessments in the immature animal is encouraged.

Introduction

- The Food Quality Protection Act (1996) instructs EPA, in making its "reasonable certainty of no harm" finding, that in "the case of threshold effects, **an additional tenfold margin of safety** for the pesticide chemical residue and other sources of exposure shall be applied for infants and children to take into account **potential pre- and postnatal toxicity and completeness of data with respect to exposure and toxicity to infants and children**."
- Section 408 (b)(2)(C) further states that "the Administrator may use a different margin of safety for the pesticide chemical residue only if, on the basis of reliable data, such margin will be safe for infants and children."

Developmental Neurotoxicity Study

- The test substance is administered daily, generally orally, to mated females (rats are preferred) from the time of implantation (GD 6) throughout lactation (PND 21).
- At least three dose levels and a concurrent control should be used and a total of 20 litters are recommended at each dose level.
- Gross neurologic and behavioral abnormalities, and the evaluation of brain weights and neuropathology during postnatal development and adulthood.



~1100 animals, often more.

Requires many months/year to conduct & analyze all aspects of the study

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OPP DNT Challenges

- Challenges on interpretation across the studies
  - Motor activity:
    - multiple types of measurements(i.e. beam breaks v. distance traveled) and quantifications make data hard to compare across compounds
    - High variability (50% or higher)
  - Morphometric data:
    - orientation of slices differs between studies and contract laboratories.
    - parameters measured (i.e. length, width, height) also differ which also makes comparison difficult
    - Learning and memory methods differ across studies
- Labs providing inadequate statistical analyses on behavioral data
- Lack of comparable methodology across studies

OPP DNT Determination

- Weight of Evidence Approach for Requiring a DNT
  - Quantitative susceptibility observed in database
  - Neurotoxicity observed or plausible
  - AOP knowledge
  - Current risk assessment endpoints may not fulfill FQPA lifestage safety finding, 10x retained if uncertainty remains

Status of DNT Studies at OPP

- Currently 101 DNT studies reviewed by OPP
- 24 DNTs currently used as points of departure
  - All 24 DNTs are based on offspring effects without corresponding maternal effects
    - Pup mortality (5)
    - Brain morphology (9)
    - Pup weight (4)
    - Behavioral changes (5)
    - Developmental delays (1)

Status of DNT Studies at OPP

- Endpoints of the 24 DNTs used as points of departure:
  - offspring brain morphology (9) and behavioral changes (5) unique to the DNT
  - pup mortality (5): effects identified in the reproduction studies
  - pup weight (4), and developmental delay (1) were also observed in the reproduction studies

Status of DNT studies at OPP

- Registration Review- a 15 year re-evaluation
  - 2 DNTs required during Registration Review
    - 1 DNT required due to unknown AOP
    - 1 DNT required but option for alternative study design; on-going
  - 13 DNT studies waived from RED requirements
    - 1 was an OP
    - 2 the liver was the target organ
    - 10 No susceptibility and no neurotoxicity or only at high doses
  - DNTs also waived for pyrethroids
  - DNTs waived for other OPs

DNT Study Alternatives: OPs

- In 1999 a data-call-in issued for DNTs for the organophosphates (OPs)
- 18 DNTs submitted for the OPs
  - -None of the DNTs used in risk assessment
- Comparative Cholinesterase Study (CCA):
  - -10% Cholinesterase (ChE) inhibition
    - Acute (juvenile and adult)
    - Repeat (juvenile and adult)
    - Gestational (fetal)

DNT Study Alternatives: OPs

- USEPA typically reviews the CCA protocol before conducted:
  - RBC and brain ChE sampled
  - Time course data to determine the time to peak effect
  - Dose selection
    - Typically 3 doses & control
    - However, sometimes 4-5 doses but limit the sample size or only 1 sex to limit animal use.
  - High quality studies
  - Nearly all registered OPs in the US have part or all of the CCA
  - Benchmark dose analysis on the studies

DNT Study Alternatives: NMCs

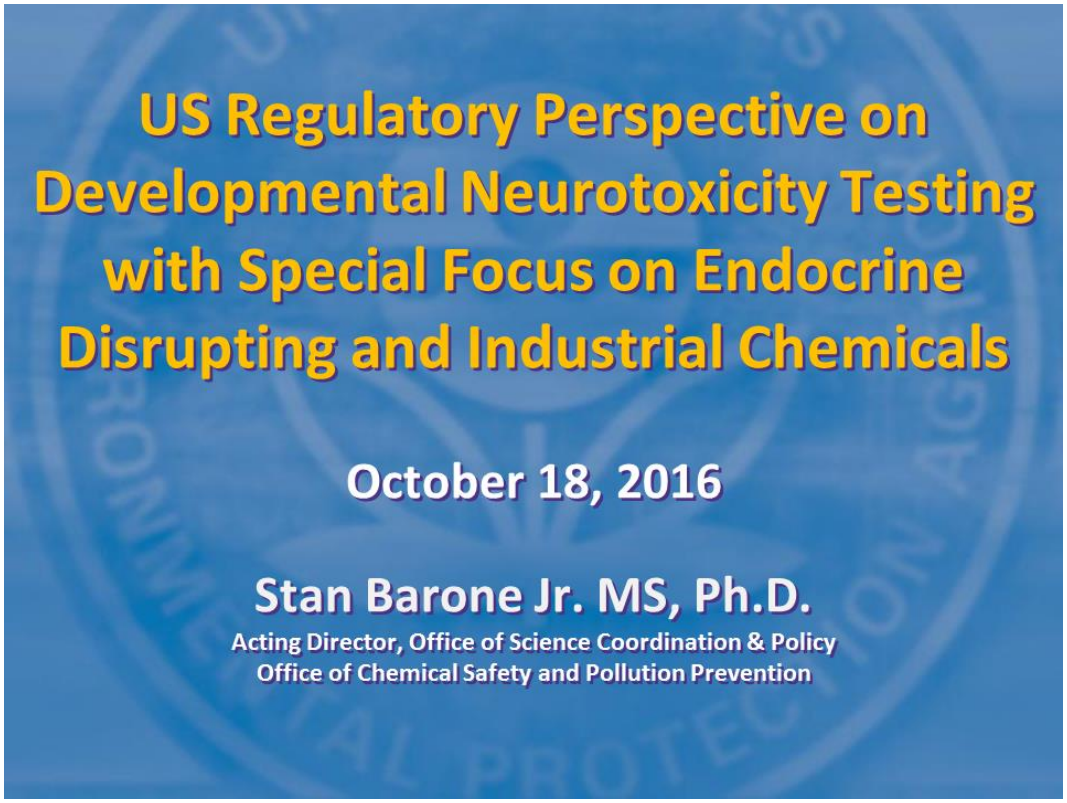
- *N*-Methyl Carbamates (NMCs)
  - DNTs available for 3 NMCs
  - CCA studies available for all 8 NMCs
  - Specifically designed based on AOP
    - RBC and brain ChE
    - Time to peak (15-45 minutes) in adults and juvenile
    - Time to recovery (minutes to hours)
    - Adult and juvenile (PND11-17)
  - High quality studies
  - Benchmark dose analysis on the studies
  - ChE data relied upon for risk assessment

Conclusions

- AOP knowledge has worked well for OPs and NMCs
- CCA studies for OPs and NMCs
- CTA studies now being called in for developmental thyroid AOPs
- Lifestage data derived differences
- In vitro/alternative assays to the in-life DNT could inform:
  - a screen for future pesticides;
  - prioritize testing of pesticides;
  - potential for lifestage susceptibility and the FQPA factor

[PPT 6] US Regulatory Perspective on Developmental Neurotoxicity Testing with Special Focus on Endocrine Disrupting and Industrial Chemicals

Stanley Barone Jr., U.S. Environmental Protection Agency (USA)



What is the problem and how big is it?

- Broad chemical space and broad biological space required for testing of potential neurotoxicants and especially developmental neurotoxicants.
- Huge number(~10K) of substances which excludes pesticides and drugs with known mode of action and target sites.
- Approaches Needed for Prioritization, Screening, Testing and Assessment.

TSCA Major Improvements in 2016

- Mandatory duty on EPA to evaluate existing chemicals with clear and enforceable deadlines
  - *Old TSCA – no duty to review; no deadlines for action*
- Chemicals assessed against a risk-based safety standard
  - *Old TSCA – risk-benefit balancing standard*
- Unreasonable risks identified in the risk evaluation must be eliminated
  - *Old TSCA – Signficant risks might not be addressed due to cost/benefit balancing and no mandate to act*
- Expanded authority to more quickly require development of chemical information when needed
  - *Old TSCA – Required lengthy rulemaking*

TSCA New Chemicals

- TSCA 21 requires EPA to make affirmative finding on new chemicals or significant new uses of existing chemicals
- Before the chemical can enter the market, EPA must find that the chemical:
  - "presents an unreasonable risk" and issue a 5(f) order to address such risk;
  - "information…is insufficient to permit a reasoned evaluation…" and issue a 5(e) order;
  - "may present an unreasonable risk" and issue a 5(e) order; or
  - is "not likely to present an unreasonable risk"

TSCA Specific Requirements Existing Chemicals

- Prioritizing Chemicals for Assessment
  - Establish a risk-based process to identify "high" and "low" priority substances
  - High priority – the chemical may present an unreasonable risk of injury to health or the environment due to potential hazard and route of exposure, *including to susceptible subpopulations*
  - Low priority – the chemical use does not meet the standard for high-priority

TSCA Alternative Testing

Section 4

When requiring the development of new information relating to a chemical substance or mixture under paragraph (2), the Administrator shall identify the need for the new information, describe how information reasonably available to the Administrator was used to inform the decision to require new information, **explain the basis for any decision that requires the use of vertebrate animals**, and, as applicable, explain why issuance of an order is warranted instead of promulgating a rule or entering into a consent agreement.

TSCA Tiered Testing

Section 4

When requiring the development of new information under this subsection, the Administrator **shall employ a tiered screening and testing process**, under which the results of screening-level tests or assessments of available information inform the decision as to whether 1 or more additional tests are necessary, unless information available to the Administrator justifies more advanced testing of potential health or environmental effects or potential exposure without first conducting screening-level testing.

What is the scope of the problem?

- Can we estimate how many chemicals in commerce are neurotoxic (NTX)?
- Best estimates-

  OTA estimates (1990) that the number chemicals (> 65,000) in commerce with NTX potential ranged from 3-28% (2,000- 20,000).

  Majority of over 500 registered pesticides have NTX mechanism/mode of action

Endocrine Disruptor Screening Program

- **Mission:** To protect public health and wildlife by screening and testing chemicals and taking appropriate actions for those chemicals that are found to have endocrine effects.

- Based on two legislative mandates:
  - 1996 Federal Food, Drug and Cosmetic Act, Section 408(p)
  - 1996 Safe Drinking Water Act Amendments, Section 1457

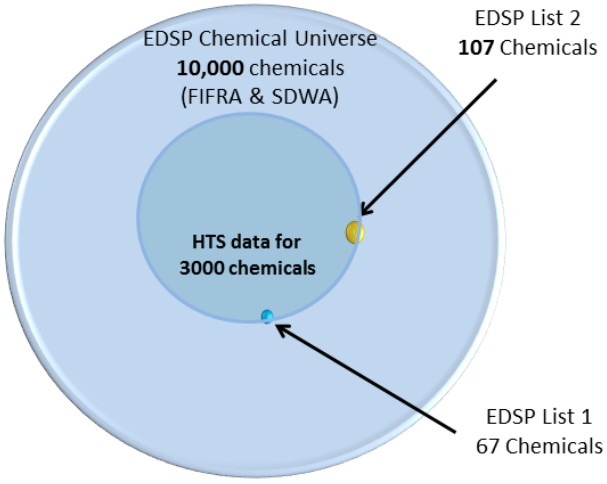
- Focus on Estrogen, Androgen, Steroidogenesis and Thyroid Pathways

- Program based on a Tiered Approach:
  - Tier 1: Screening to identify chemicals that have potential to interact with the endocrine system using a battery of assays
  - Tier 2: If found to have potential, then Tier 2 testing may be required to identify and establish doses at which adverse effects may occur

EDSP Universe of Chemicals

Chemical List	Number of Compounds
Conventional Active Ingredients	838
Antimicrobial Active Ingredients	324
Biological Pesticide Active Ingredients	287
Non Food Use Inert Ingredients	2,211
Food Use Inert Ingredients	1,536
Fragrances used as Inert Ingredients	1,529
Safe Drinking Water Act Chemicals	3,616
TOTAL	**10,341**

Evolution of EDSP- the "Pivot"



- Based on current pace it could take decades to screen all 10,000 chemicals in EDSP Universe
- Employ high throughput assays and predictive models to rapidly screen chemicals for potential bioactivity and exposure

Is Assessment of Developmental Neurotoxicity Necessary?

- Do we assess the structural and/or functional integrity of the nervous system following developmental exposure in Multigen/Extended 1 Gen or Developmental Guideline studies.

- If you don't look you don't see (Goldey *et al.*, 1995; Ulbrich and Palmer, 1996, Makris *et al.*, 1999, DNT Retrospective study).

NTX related testing

- EDSP Tiered screening recommendations for CTA (comparative thyroid assay) 4 pesticides from EDSP list 1
- Pesticide actives -101 DNT data evaluation records (DERs) with 24 DNT endpoints serving as regulatory endpoints
- Requests for TSCA new chemicals NTX testing since 1979-2016---1,010 consent orders covering 1,666 PMNs out roughly 22,000 PMNs- ---testing triggered
- 5 out of 55 New chemical categories identify NTX as CE possible NTX testing; 2/5 developmental NTX
- NTX for existing chemicals not required but one of screening criteria in OPPT work plan was NTX potential

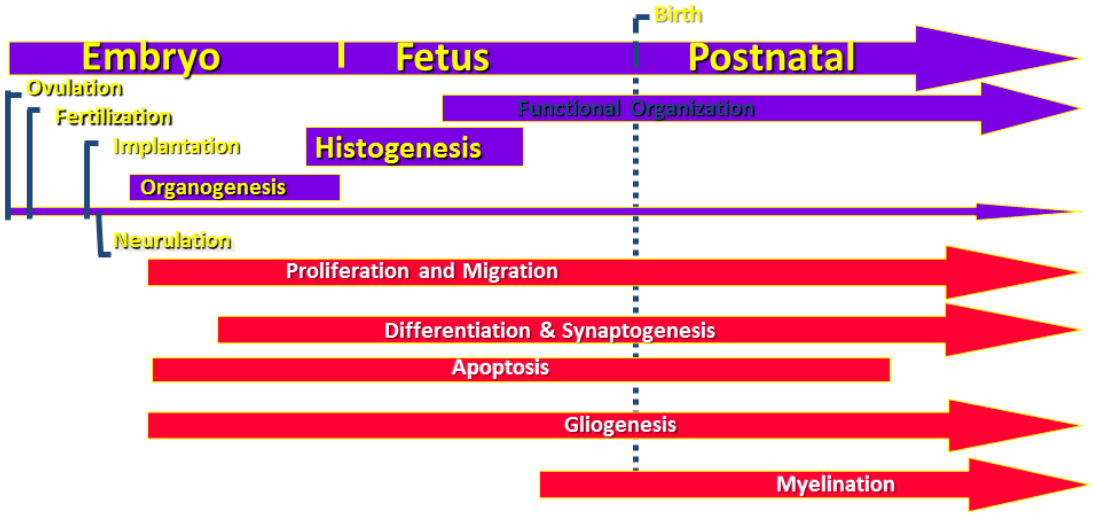
Summary of NTX in IRIS data base of Existing Chemicals (Tilson, 2000)

Sufficient data base for NTX	**392**
• Critical effect (CE) was NTX	74
– DNT endpoint critical effect	2 out of 74
• NTX reported but not CE	46
• Non NTX	272
Insufficient data base for NTX	**145**
• Cancer assessments	57
• Noncancer assessments	1
• No values listed	87
TOTAL	**537**

Challenges in Developmental Neurotoxicology

- **Complexity of Nervous System.**
  - **number of cells.**
  - **number of cell phenotypes.**
  - **number of connections.**
- **Baseline may change rapidly with Time.**
  each region has different time scale.
- **Patency of blood-brain barrier limited during early development**
- **Potential for Compensation and Recovery.**
- **"Silent damage"**
  - **Not expressed till later in life or**
  - **Not revealed until challenged**

Development is Temporally and Regionally Determined by Multiple Processes.



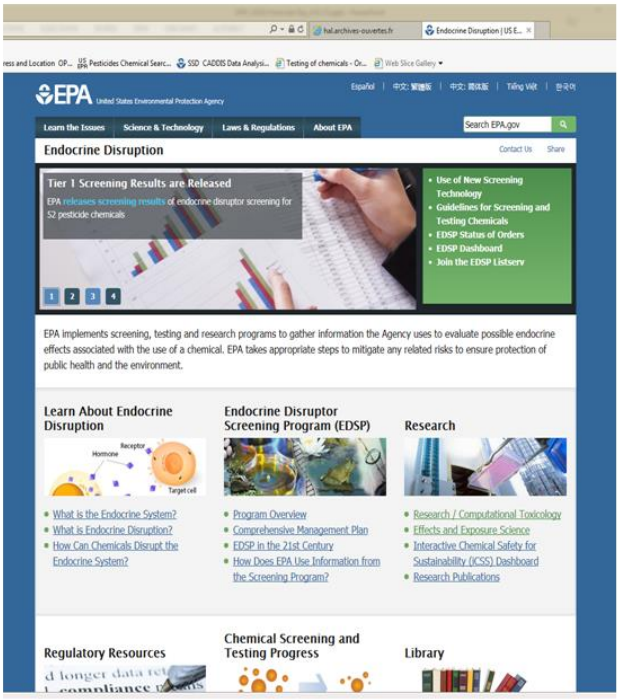
Barone, S. Jr. *et al.*, 2000; *NeuroToxicology*. 21:(1-2) 15-36.

DNT Testing Battery Needs

- Screening of high volume of chemicals
- Coverage of a broad number of biological processes and targets
- Well characterized performance based criteria for test of battery
- Reference list of chemicals for tests
- Predictive of adverse outcomes
- Informative of chemical categories, SAR and QSAR for prioritizing further testing

Summary of Use of NTX and Developmental NTX in Decision Context

- Screening and prioritization of thousands of chemicals
- Testing of chemicals based upon insight of AOPs- potential to reduce number of animals used in testing
- Setting up biological context for read across approaches using AOP's and HTS
- Informing weight of evidence analysis
- Inform risk decision using SAR, QSAR, read across approaches in New Chemicals context



https://www.epa.gov/endocrine-disruption

Acknowledgements

- EPA OSCP/OPP
- EPA ORD

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  - http://www.wws.princeton.edu/~ota/ns20/year_f.html
- OTA, 1995: *Screening and Testing Chemicals in Commerce* (Chapter 4)
  - http://www.wws.princeton.edu/~ota/ns20/year_f.html

Recommended Additional Testing including TIER 2 for Chemicals that Showed Potential Interaction with E, A and/or T

	Chemical	Human Health	Wildlife
1	Carbaryl	None	MEOGRT
2	Chlorothalonil	None	LAGDA
3	Cypermethrin	Special study: Assess androgen-related effects in adult males	MEOGRT
4	DCPA	CTA (comparative thyroid assay)	LAGDA
5	Dichlobenil	None	MEORGT
6	Dimethoate	CTA	None
7	Flutolanil	None	MEOGRT
8	Folpet	None	MEOGRT
9	Iprodione	None	MEOGRT
10	Linuron	CTA	MEOGRT, LAGDA
11	Metalaxyl	None	MEOGRT
12	Metribuzin	CTA	LAGDA
13	Myclobutanil	None	MEOGRT
14	O-phenylphenol	None	MEOGRT
15	PCNB	None	MEOGRT
16	Propargite	None	LAGDA
17	Propiconazole	None	MEOGRT
18	Tebuconazole	None	MEOGRT

EDSP "Pivot" Announcement

FEDERAL REGISTER
The Daily Journal of the United States Government

June 19, 2015
FRL-9928-69

"Use of High Throughput Assays and Computational Tools; Endocrine Disruptor Screening Program; Notice of Availability and Opportunity for Comment"

https://www.federalregister.gov/articles/2015/06/19/2015-15182/use-of-high-throughput-assays-and-computational-tools-endocrine-disruptor-screening-program-notice

[PPT 7] EU Industry Perspective: Emphasis on Pesticides
Gaby Schmuck, representing European Crop Protection Association (BEL)

Science For A Better Life

EU Industry Perspective

Emphasis on Pesticides

- Gabriele Schmuck

Agenda

- Regulatory requirements
- Current approach
- Opportunities
- Need for alternatives
- Available test systems and the stony way to accepted models
- Conclusions

Regulatory Requirement (emphasis on pesticides)

- EFSA: “Risk based Approach”
  - Comm. Reg. 283/2013: 5.6.2. Developmental toxicity studies

    "When indicated by observations in other studies or the mode of action of the test substance, supplementary studies or information may be required to provide information on the postnatal manifestation of effects such as developmental neurotoxicity.“
- ECHA: „Hazard based Approach“
  - Trigger for DNT cohort in OECD TG443

    "Information on developmental neurotoxicity and developmental immunotoxicity are not standard information requirements in REACH but they must be proposed when particular concerns as specified in Column 2 are met.!“
    - Existing information on the substance (or structural analogue)
    - Mode of Action: neurotoxicity
    - Mode of Action: endocrine / thyroid activity

Current approach

- Acute and repeated-dose studies suggest neurotoxicity
- Developmental toxicity studies in rat and rabbit; €600,000 (80-100 litters)
- Multi-generation reproduction study (dietary); €650,000 (80-100 litters F1 & F2)
- All trigger:
  - DNT study in rat (pesticides); €750,000 (80-100 litters)

  or
  - Extended one-generation reproduction study in rat (chemicals); €1.2 M (80-100 litters; one or two generations)
- Intensive use of animals and resources

Opportunities

- Current uses of *in vitro* systems by industry
  - Skin sensitization – 3 test systems currently recognized by ECVAM
  - Skin and eye irritation; Skin corrosion
  - Genotoxicity testing (Ames, HPRT, MNT, etc.)
  - Endocrine Disruption
    - Battery of *in vitro* and simple *in vivo* tests: estrogenic, androgenic, thyroid pathways
    - Established assay performance criteria
    - Reliability shown with reference chemicals (positive and negative controls)
    - Refine or replace assays based on "track record" (e.g., incidence of false-positive)
- No validated test system for neurotoxicity and developmental neurotoxicity available

Need for Alternatives

- Test systems with predictive reliability to identify hazards that are relevant to human health.
  - Candidate selection
    - Quick and predictive test systems
    - Capacity for higher throughput
  - Decision making in development
    - Better understanding of findings (e.g. in in vivo studies)
    - MoA
    - More mechanistic based approaches
  - For market authorization:
    - Relevance for humans
    - Safety factors

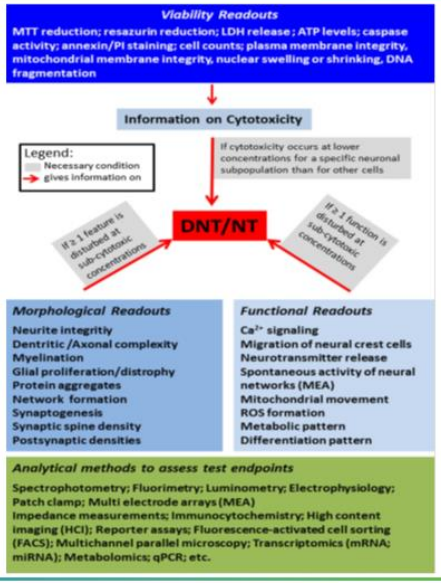
In vitro Assays

- Available test systems (examples)

Model	Strength	Weaknesses
Permanent cell lines	Available from cell bank	Physiology questionable; derived from tumors
Non- human		
Primary rodent cells	Functional neuronal circuits	New animals needed for each culture, labor intensive
Mouse embryonic stem cells	Pluripotent, established	Non human physiology
Human		
Embryonic stem cells	Neuronal network, self-renewing, pluripotent, differentiation protocols available	Culture expensive and protocols may be lengthy, originate from single sources (history/ genetic background not known)
Induced pluripotent stem cells	Neuronal network, self-renewing, pluripotent, differentiation protocols available	Culture expensive and protocols may be lengthy, variable from line to line (genetic background not known)
3 D cultures	Tissue self-organization, differentiation, migration	Complex analysis, difficult quality control

In vitro Assays

- Available Read outs (examples)*



*(from Schmidt, et al. 2016)

Exposure in vivo

- Risk assessments for human safety require knowledge about exposure
  - Exposure route for humans: consumer, operator (e.g. dietary, dermal, inhalation)
  - Is knowledge about the exposure in the animal/ humans?
  - Is substance metabolized, do metabolites play a role in neurotoxicity?
  - Can substance cross BBB or placental barrier?
  - Can substance be excreted via milk?
  - Plasma protein binding (usually lower protein amount in cell culture!)

Exposure in vitro / Quality control

- Exposure in the test system
  - Continuously or for certain time periods
  - Medium change
  - Does the chemical partition (concentrate) in the cells or tissue?
  - Metabolic systems added
  - Barrier systems available
  - Physico-chemical properties known
- Quality control of exposure
  - Knowledge of concentration and stability of the test substance in solvent?
  - Knowledge of concentration and stability of the test substance in medium?
  - Affinity to plastics in tissue dish or tubes

Test system

- Established cell system?
  - Reproducible (e.g. composition of cell types, age of culture, genetic variability…)
  - Quality control (visual, functional)
  - Suitable for the defined endpoint?
- Endpoints
  - Reproducible (like under GLP: equipment like pipettes, incubator gauged?)
  - Quality control with positive and negative substances
    - Problematic, because not many dev. neurotoxins known aside from heavy metals
  - Can endpoints discriminate neurotoxicity from developmental neurotoxicity?

Conclusions

- **There is a high need for alternative methods for developmental neurotoxicity testing**
  - Candidate selection, decision making, MoA exploration
- Large variety of test systems and endpoints are available
  - Which is suitable for what (mechanistically based)?
  - Which is suitable in regard to higher throughput?
  - Which fits into the regulatory required testing strategy (risk assessment)?
  - Which is predictive for humans?
- Risk assessment
  - "validated" system?
    - One system could not address all questions
  - Integrated Approaches to Testing and Assessment (IATA)
    - Knowledge from in vivo studies (incorporate endpoints into routine tox. studies, using existing knowledge)
    - Knowledge from in vitro studies

[PPT 8] U.S. Industry Perspective: DNT Testing Strategies Based on Alternative Assays
Sue Marty, DOW (USA)

U.S. Industry Perspective: DNT Testing Strategies Based on Alternative Assays

Sue Marty, Ph.D., DABT
TERC Science Leader
The Dow Chemical Company

The Human Element at Work.

Agenda

- Industry view of alternative assays
  - Why use alternative approaches?
  - Current use of alternative models
  - Integrating assay data with exposure
- *In vivo* approaches to evaluate Neurotoxicity/DNT
- Alternative DNT approaches and their challenges
- Characteristics of DNT Test Systems
- The Exposure-Effect Discontinuum
- Conclusions

Why Use of Alternative Approaches?

- *In silico*, *in vitro* and alternative *in vivo* models
- Industry supports alternative approaches to assess toxicity, including DNT:
  - Initial hazard characterization (early in development)
  - Prioritize testing based on bioactivities of potential concern
  - Select R&D candidate compounds
  - Test formulations
  - Used for read across
  - Targeted toxicity testing
  - Generate more complete toxicity evaluations
  - Evaluate potential MOA (mode of action)

DOW

The Human Element at Work.

Current List of Alternative Models

Predictive Toxicology Approaches (Mammalian/Environmental)
Cheminformatics (*in silico* models) • QSAR • Analog ID & Read across • Metabolism modeling • Systemic exposure
Exposure Modeling • HTP • IVIVE
Biological Profiling (*in vitro* approaches) • Dermal/ocular corrosion and Irritation • Skin sensitization • Phototoxicity • Respiratory irritation • Mutagenicity • Endocrine Activity • DART • Toxicogenomics • Oxidative Stress • PBT • BCF • *In vitro* TK • Microplate acute ecotox screening

Alternative Models must be "fit for purpose" (e.g., are results for prioritization vs. regulatory submission?)

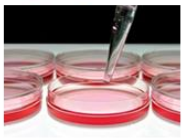
DOW

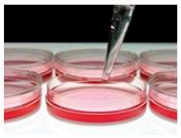
Integration: Assay Data + Exposure (IVIVE)

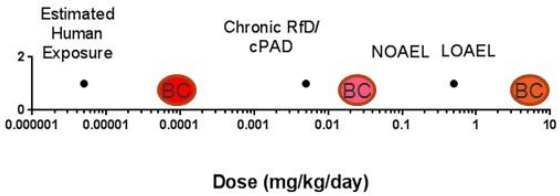
Predict exposure producing bioactive concentration of compound at the target site

Rat *in vitro* data to predict *in vivo* exposure…

Human PBPK + *in vitro* data to predict human dosimetry







DOW

The Human Element at Work.

In vivo Approaches to Evaluate Neurotoxicity/DNT

- Testing requirements for industrial chemicals depend on tonnage produced (REACH), use/potential for exposure, NT signs, etc.

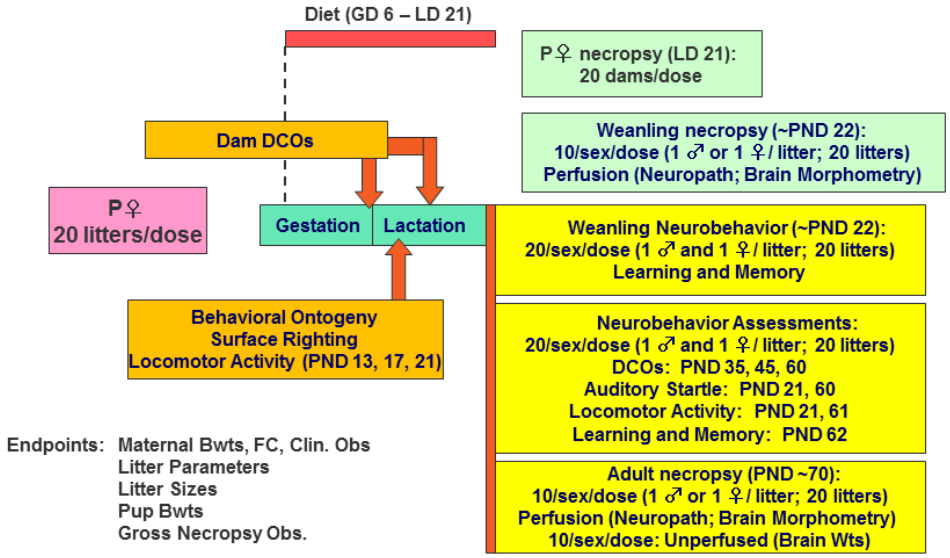
In vivo Approaches for Neurotoxicity and DNT

- Acute toxicity/neurotoxicity studies
- Repeat-dose toxicity/neurotoxicity studies (28-day, 90-day)
- Repro/devtl/repeat-dose screening assays (OECD 421/422)
- Developmental toxicity studies (OECD 414)
- Endocrine effects (e.g., thyroid – repeat-dose, OECD 421/422, pubertal assays, etc.)
- Two-generation (OECD 416) or EOGRTS (OECD 443)
- Developmental neurotoxicity study (OECD 426)
- Neurotoxicity target is unknown (need to detect a spectrum of effects)
  - Neurobehavioral assessments to evaluate integrated NS function
  - Neuropathology

DOW Labor and Resource Intensive

The Human Element at Work. 6

DNT Study (OECD 426)

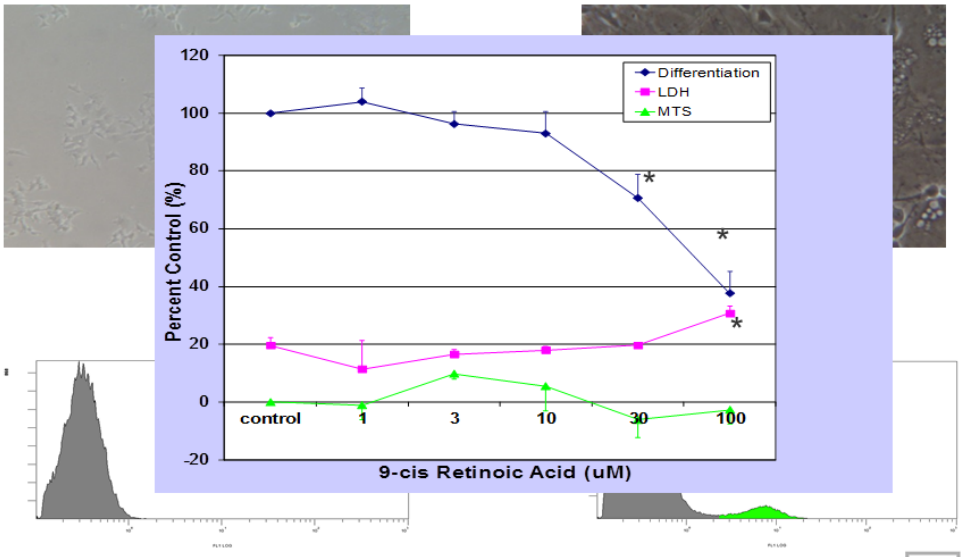


- Exposure to offspring confirmed with PK
- Retrospective analysis: DNT solely determines RfD ~5% (US EPA)

Alternative Assays to Detect DNT

- Assays should include effects specific for DNT (e.g., neuronal proliferation, migration, differentiation, myelination, etc.)
- Numerous alternative models in development:
  - Primary cells: neurons and glia from different brain regions
  - Neural stem (progenitor) cells
  - Cell lines: neuroblastoma, astrocytoma, glioma,
  - Organotypic (3D) co-cultures
  - Organisms: *C. elegans*, Zebrafish
- Detection methods:
  - Multi electrode arrays
  - Neurite outgrowth, Neurodegeneration, Cell proliferation, Apoptosis
  - Calcium flux
  - Synaptogenesis
  - Behavioral changes
- Need a battery approach/an integrated system that can detect multiple MOAs

Neuronal Differentiation with NT2 Cells



DOW

The Human Element at Work.

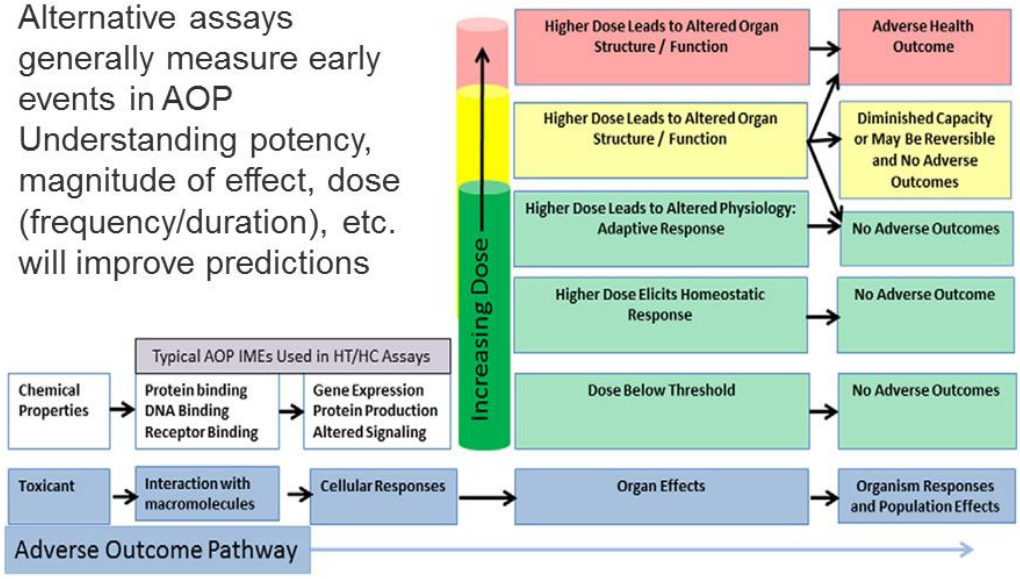
Characteristics of DNT Test Systems

- Bioprofiling…confidence is key!
  - Validity: relevance, reliability, sensitivity, specificity of assays
  - Regulatory agencies, regulated community and public must be confident
- Test systems should include:
  - Rationale and purpose for method
  - Relationship of test endpoint(s) to biological effect of interest
    - ID hazards relevant to human health (adverse effect that is biologically-plausible at relevant concentrations)
  - Detailed protocol
  - Chemical domains of applicability
  - Criteria for data interpretation (prediction model)
  - Assay limitations (e.g., in vitro metabolism/ADME, cell stress/cytotoxicity, non-specific effects, potency predictions)
  - Procedures to ascertain method performance (pos/neg controls for reproducibility; performance criteria, sensitivity; etc.)

DOW

The Exposure-Effect Discontinuum

- Alternative assays generally measure early events in AOP
- Understanding potency, magnitude of effect, dose (frequency/duration), etc. will improve predictions



Patlewicz et al., Reg. Toxicol. Pharmacol. 65:259, 2013.

Conclusions

- Alternative approaches will allow for more rapid screening and prioritization of compounds that show DNT potential
- Assays should be "fit for purpose" and results should be evaluated in the context of exposures
- Adequate evaluation of DNT potential may require a battery of assays
- Assay characterization will improve utility and scientific confidence to predict in vivo effects.
- Continued development of AOPs is needed with a focus on understanding key event relationships.

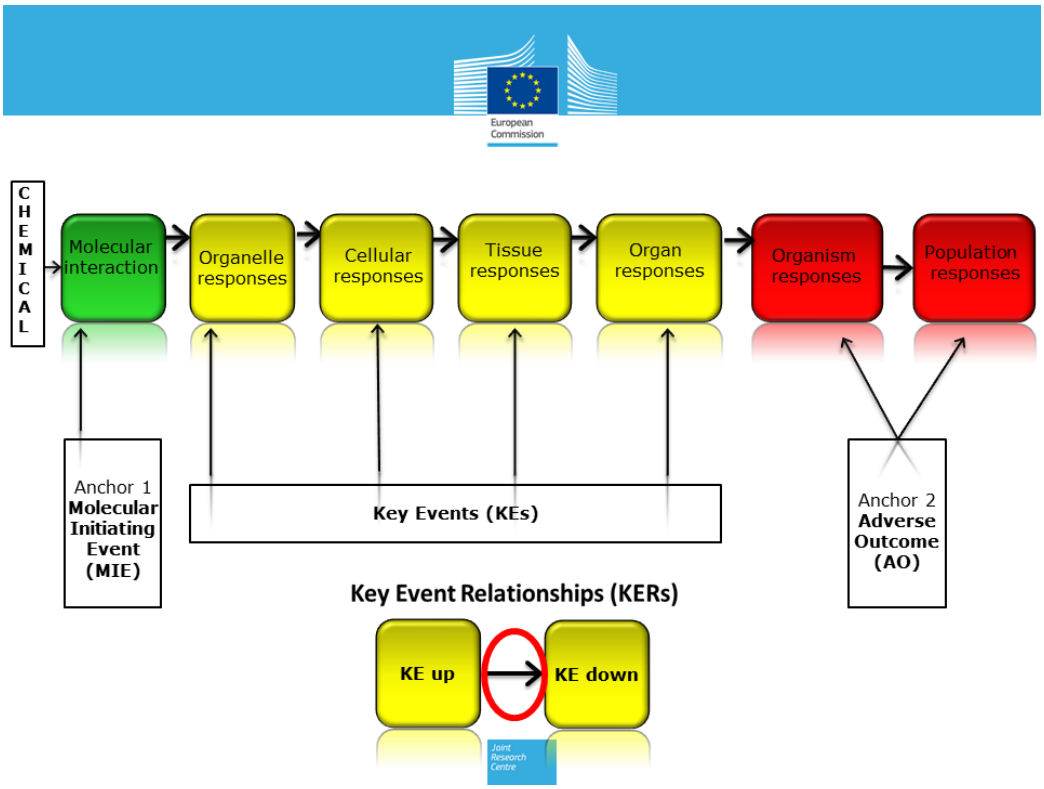
[PPT 9] Perspectives on how the Adverse Outcome Pathway concept informs the use of in vitro DNT data for regulatory purposes

Anna Price, European Commission Joint Research Centre (JRC)

Adverse Outcome Pathways - AOPs

- Out of isolated Events a Pathway emerges: **Adverse Outcome Pathway** (AOP)
- An AOP is a conceptual framework that portrays **existing knowledge** 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 molecular initiating event and an adverse outcome, 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)

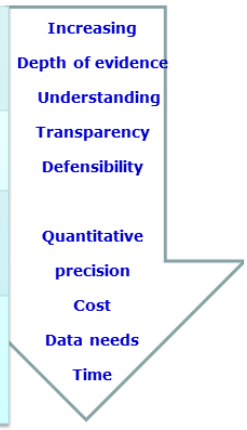
Modified Bradford-Hill Considerations	Conclusions
Biological Plausibility	KER is consistent with current biological understanding plausible.
Essentiality of Key events	Effects are reversible if the stressor is removed (e.g., Villeneuve et al. 2009; EHP 117: 624-631)
Concordance of Empirical Observations	**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

Stages of AOP Development	Characteristics
Putative AOPs:	Hypothesized set of KEs and KERs primarily supported by biological plausibility and/or statistical inference
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 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



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

AOP applicability in regulatory context:

1. **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 adverse (toxicologically relevant and predictive of the AO) from those that are adaptive (e.g. related to compensatory mechanisms that do not lead to AO).

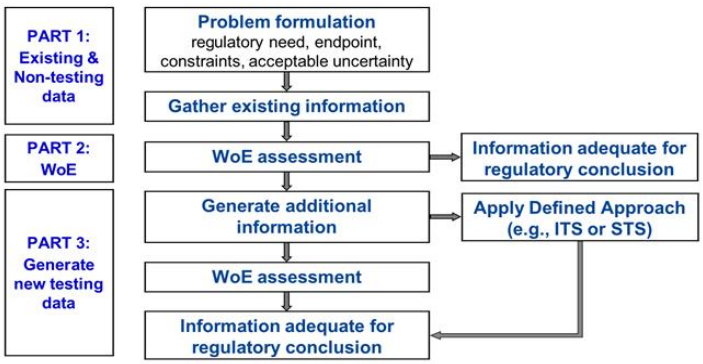
10. Inter-species extrapolation: conserved KEs within and across taxa enable identification of susceptible species

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

IATA Workflow



- 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.

4 November 2016 — *Modified IATA workflow diagram (OECD, 2016)* — 10

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

For Official Use ENV/JM/HA(2015)7

Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

Cancels & replaces the same document of 19 May 2015

Task Force on Hazard Assessment

GUIDANCE DOCUMENT ON THE REPORTING OF DEFINED APPROACHES TO BE USED WITHIN INTEGRATED APPROACHES TO TESTING AND ASSESSMENT

22 January 2016
Paris, France

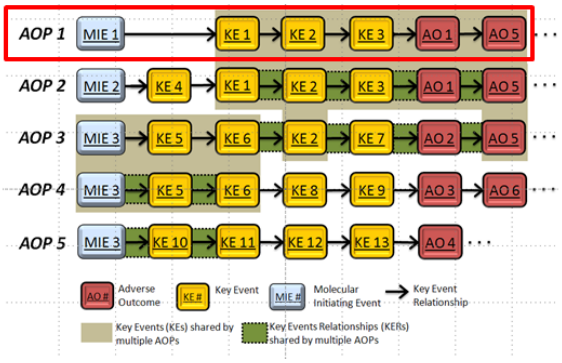
ENV/JM/HA(2015)7
For Official Use

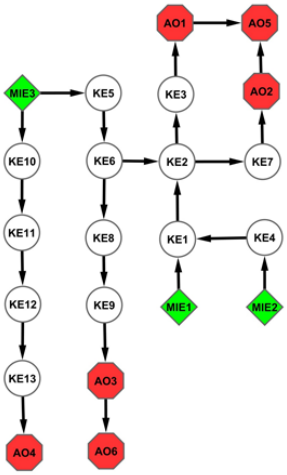
Could already available DNT AOPs serve as a guide for the selection of the most relevant in vitro assays for screening purposes to identify chemicals with DNT potential ?

Network of AOPs

European Commission

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





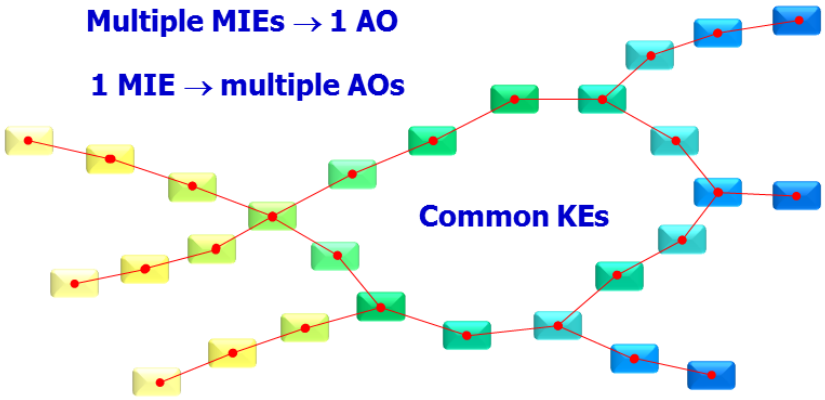
Joint Research Centre

European Commission

DNT AOPs Networking

Multiple MIEs → 1 AO

1 MIE → multiple AOs



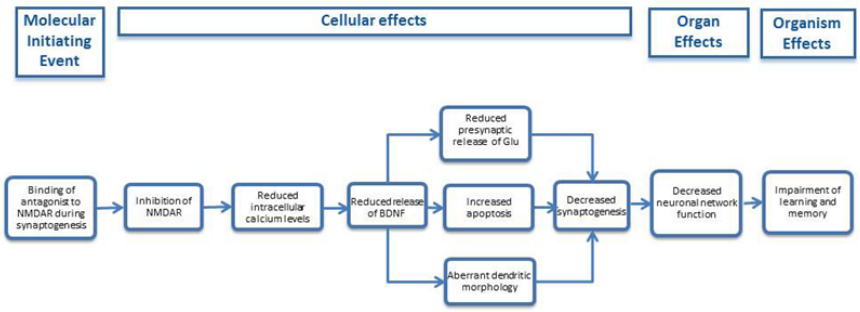
Joint Research Centre

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*)

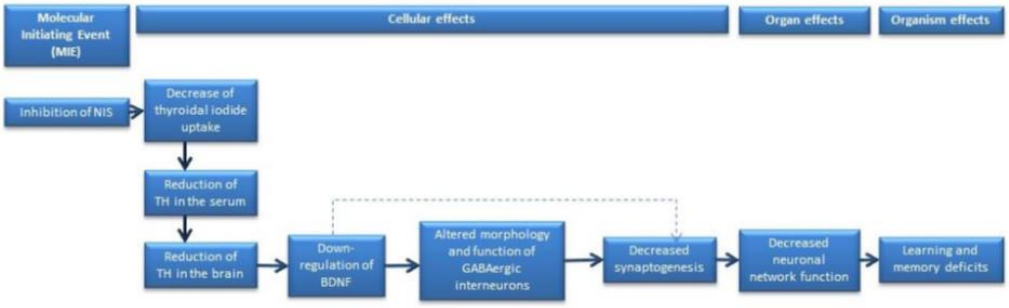


AOP-Wiki (https://aopkb.org/): Endorsed by WNT and TFHA (May 2016)

16

European Commission

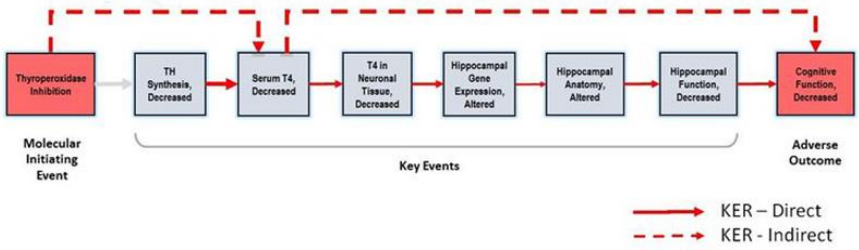
AOP 2: INHIBITION OF NA+/I- SYMPORTER (NIS) DECREASES THYRIOD HORMONE SYNTHESIS LEADING TO LEARNING AND MEMORY DEFICITS IN CHILDREN (*JRC*)



4 November 2016 Joint Research Centre *AOP-Wiki* 17

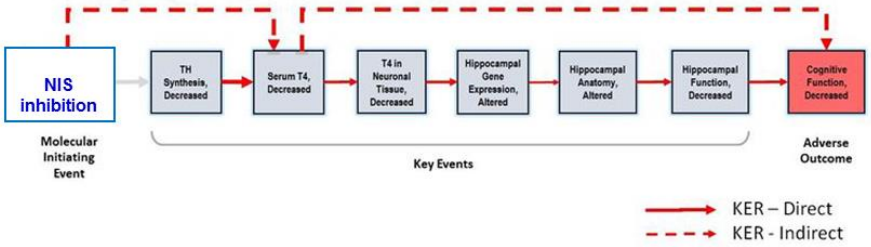
European Commission

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



4 November 2016 Joint Research Centre *AOP-Wiki (https://aopkb.org/)*

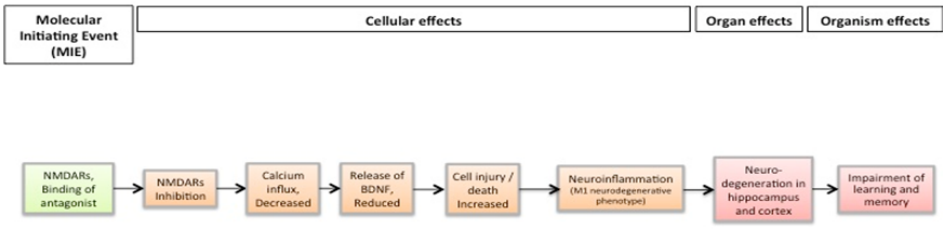
AOP 4: SODIUM IODINE SYMPORTER (NIS) INHIBITION AND SUBSEQUENT ADVERSE NEURODEVELOPMENTAL OUTCOMES IN MAMMALS (*US EPA*)



4 November 2016

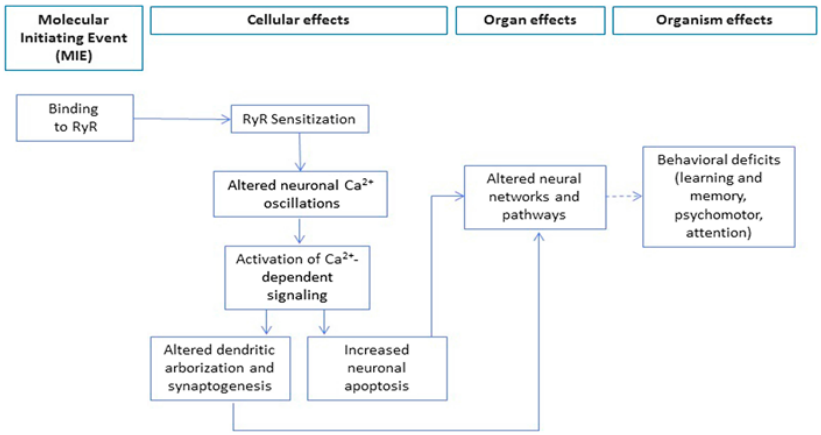
AOP-Wiki (https://aopkb.org/)

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



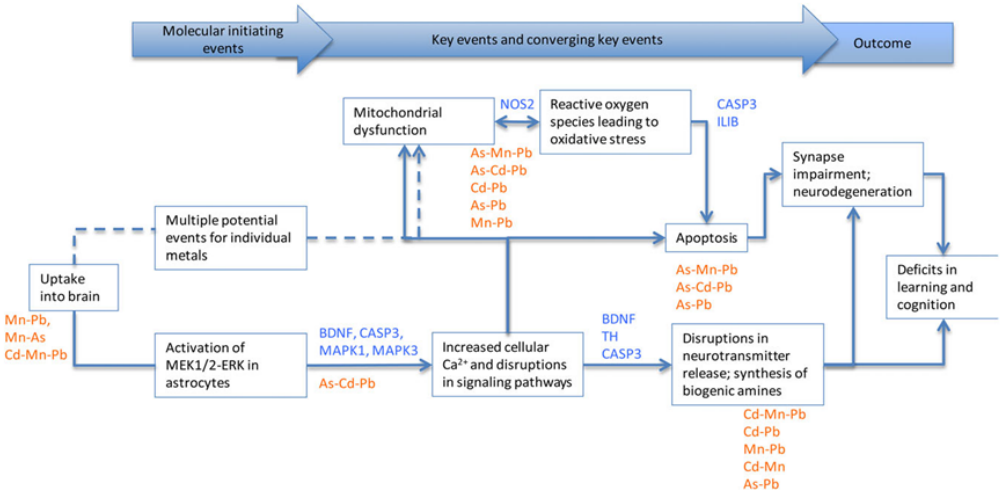
AOP-Wiki (https://aopkb.org/)

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*)



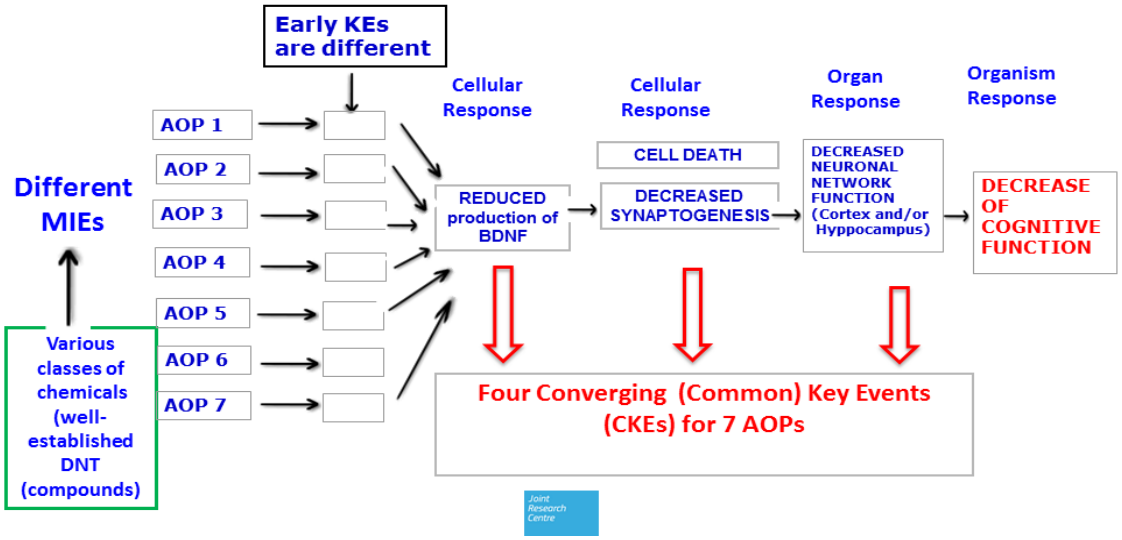
Bal-Price et al., Crit Rev Toxicol 2015

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

Common Key Events (CKEs)	*In vitro* Assays that permit to evaluate CKEs
REDUCED PRODUCTION of BDNF	BDNF protein and mRNA levels
NEURONAL and/or GLIAL CELL DEATH	Multiple techniques for specific cell types: • necrosis • apoptosis
DECREASED SYNAPTOGENESIS	• Co-labelling of synaptophysin I with PSD95 • Quantitative analysis of excitatory and inhibitory synapse • Quantitative analysis of neurite outgrowth(length and number)
DECREASED NEURONAL NETWORK FUNCTION (Cortex and/or Hippocampus)	Evaluation of network ontogeny by measuring spontaneous and evoked electrical activity in neural networks grown on microelectrode arrays

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.)

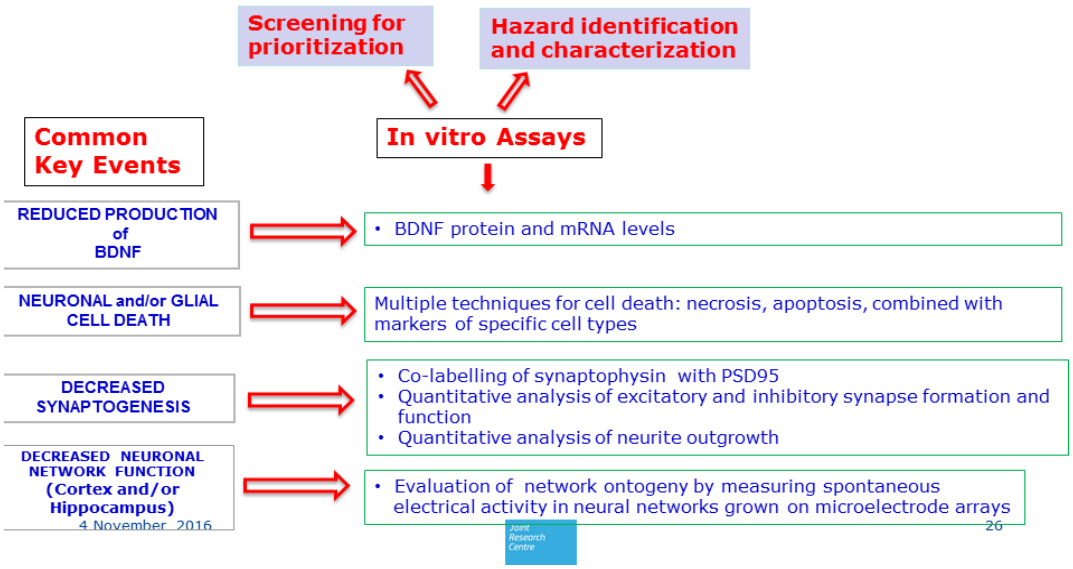
4. Co-labelling of synaptophysin with PSD95 (HCA, e.g.Thermo Fisher Scientific kit).

5. Quantitative analysis of excitatory and inhibitory synapse formation and function (Cellomics, immuno-cytochemistry, HCA).

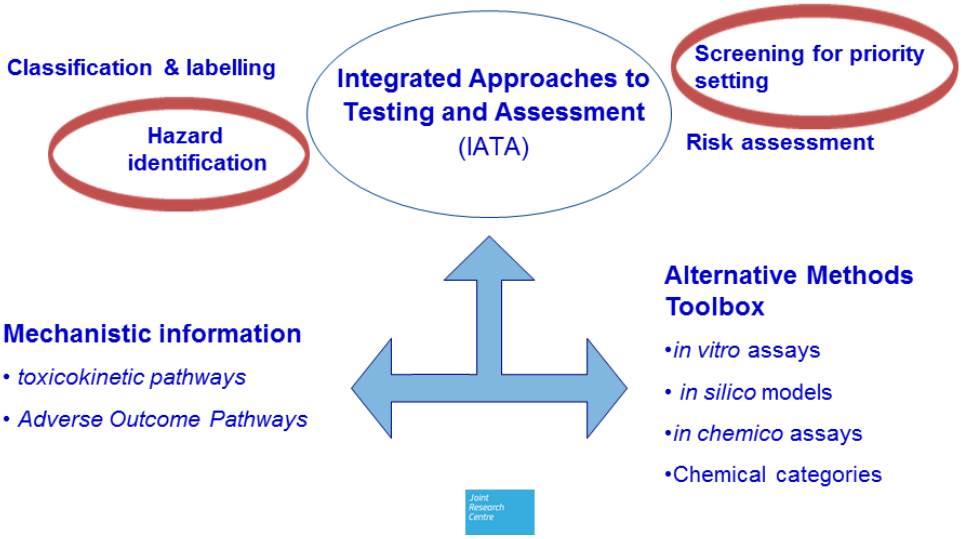
6. Quantitative analysis of neurite outgrowth (HCA and HTS: length, number etc.).

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*



AOP-informed IATA applicability in regulatory context:



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

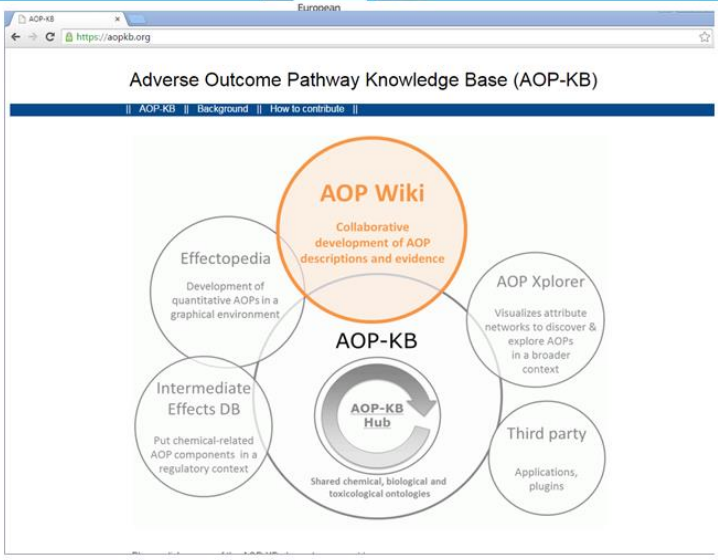
1. Further development of DNT AOPs taking into account the **temporal and dose dynamics** critical for **neurodevelopmental processes** and **key DNT signalling pathways is necessary.**

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.

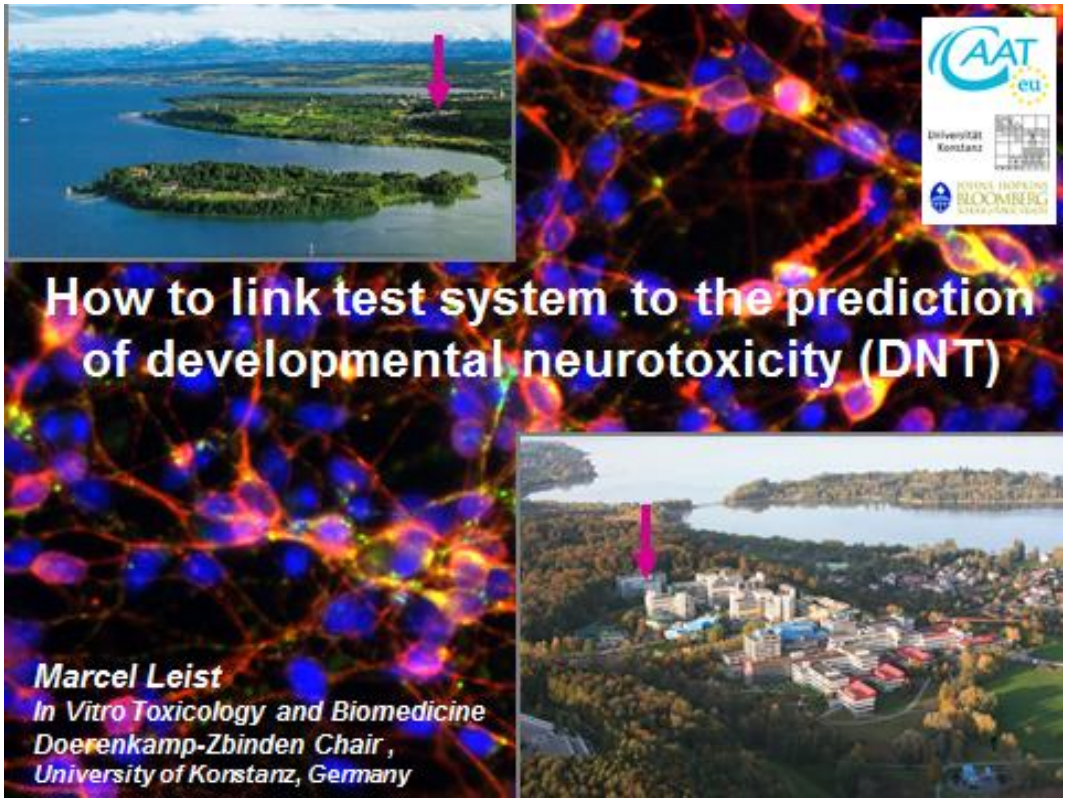
4. Develop **DNT predictive models** based on large *in vitro* data set produced by the most reliable and reproducible HTS assays.

5. Build AOP-informed IATA(s) for defined, different regulatory purposes.



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

[PPT 10] How to link test systems to the prediction of developmental neurotoxicity (DNT)
Marcel Leist, University of Kostanz (DEU)



Illustrations

Different phenotype, same brain changes...:

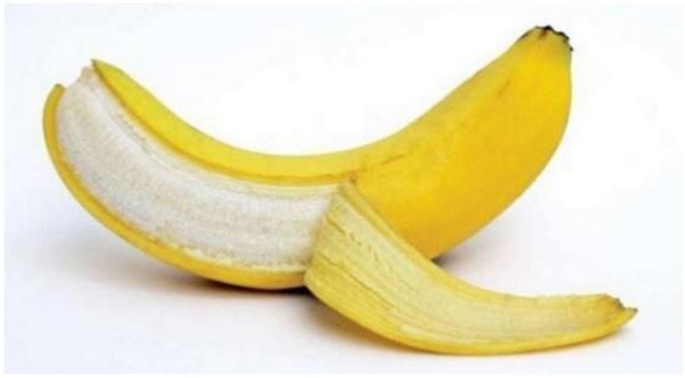
Augmented basal ganglia dopamine release (amphetamine):
Hyperactivity in **animals**
Psychosis in **humans**

Same phenotype (outside), different brain changes...:

Altered brain weight in **animals** due to loss of neurons
Altered brain weight in **humans** due to reduced gliogenesis and neurite growth

Blindness in **animals** due to toxicity to retinal ganglia cells
Blindness in **humans** due to reduced blood supply to optic nerve

Shoud we look at wrapping (outside) or at content (endogenous change)?



Can we learn from psychiatry/neurology research?

(Research area > 1000-fold larger than DNT research)

Disease:

Disease symptoms can look very similar on the outside
(→ **phenotype, exophenotype**),
although they have, e.g. in genetic diseases, entirely different causes and internal changes
(→ **endophenotype**)

Models:

Models can from the outside look very similar to the disease
(→ **face validity**),
Models can refer to a similar internal/mechanistic working
(→ **construct validity**)

Conclusion (II) from brain sciences:

The biological changes ‚**inside the brain**' are an anchoring point to define a disease: They are called **endophenotypes**.

Models with construct validity refer to **comparable endophenotypes**, and allow comparisons between disease model and human disease

In toxicology:
predictive models reflect human-relevant **toxicity endophenotypes**.
The exophenotypes (reduced verbal memory, diminished executive functions, social anxiety etc.) are hard to model.

Conclusion (II) from brain sciences:

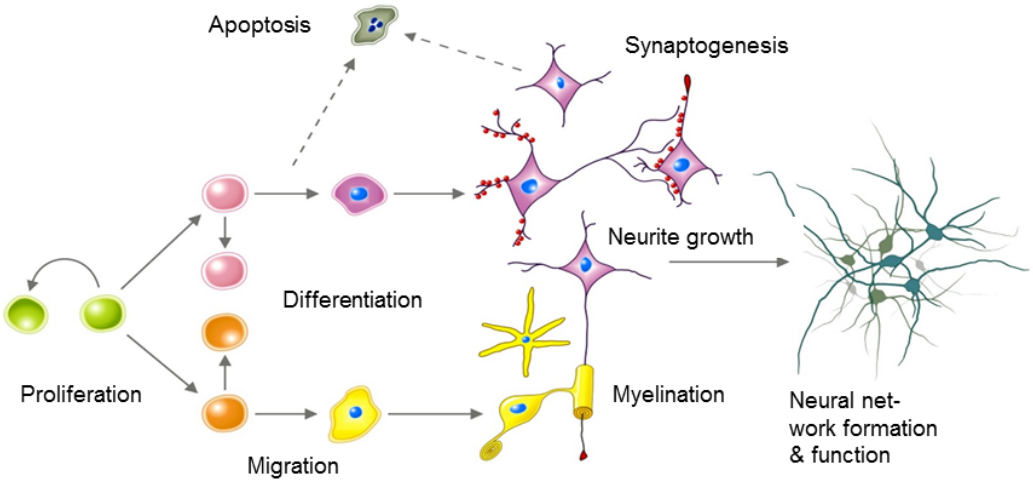
In toxicology:
predictive models reflect human-relevant **toxicity endophenotypes**.

The exophenotypes (reduced verbal memory, diminished executive functions, social anxiety etc.) are hard to model.

What determines normal or non-normal brain structure (functional or structural connectivity)?

Right or wrong:
defined by the **integrity of the processes** leading
to the final state

Any toxicity endophenotype is the result of disturbances of one or more fundamental neurodevelopmental processes



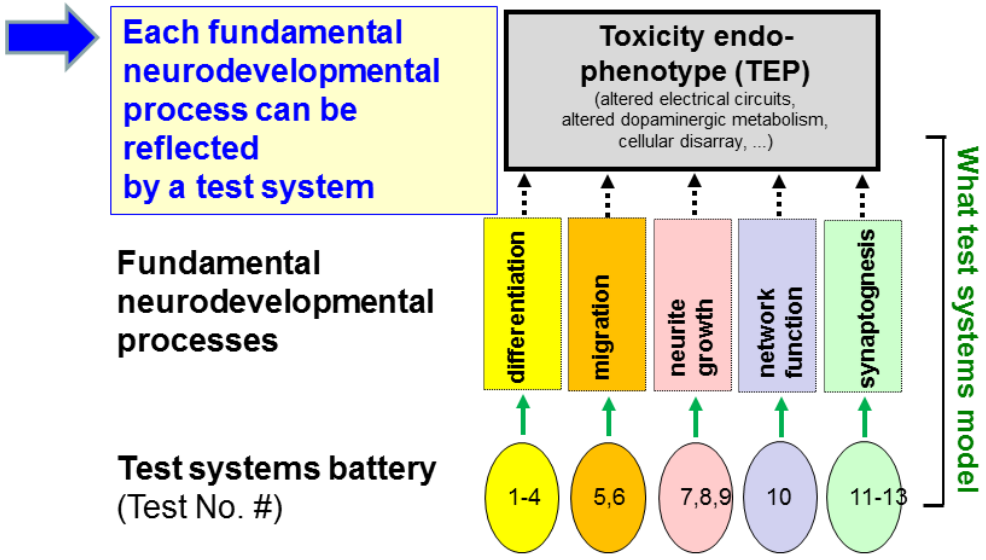
Aschner et al. (2016) ALTEX, in press

Eventually, any DNT finding (man or animal) must be due to a combination of disturbed neurodevelopmental processes

If a compound does not disturb at least one process, it cannot be associated with a DNT hazard

In vivo Finding	Disturbed neurodevelopmental processes
Brain weight up/down	Proliferation, Apoptosis
Holoprosencephaly	Apoptosis, Neurodifferentiation
Lissencephaly	Apoptosis, Neurodifferentiation, Migration
Neuroinflammation	Astrocyte activation, Gliosis, Neurodegneration
Cortical layer thickness	Proliferation, Migration, Myelination
Disturbed reflexes	Neurodifferentiation, Myelination, Synaptic transmission
Anxiety behaviour	Neurodifferentiation, Synaptic transmission, Synapse formation

Toxicity endophenotypes (TEP) are linked to human and animal outcomes. How are test systems linked to TEP?



Lessons from the development of biologics
(Erytropoietin, vaccines, blood factors,...)

End control:
The final product cannot be sufficiently controlled / described

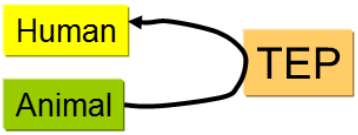
Process control:
If every production step is OK, then end product is OK.

➡ **Process control for DNT hazard evaluation: measuring whether a compound disturbs any of the key neurodevelopmental**

John Meynard Keynes:

‚The difficulty lies, not in the new *ideas*, but escaping the *old* ones'

Old idea:
Prediction of safety from undisturbed phenotype

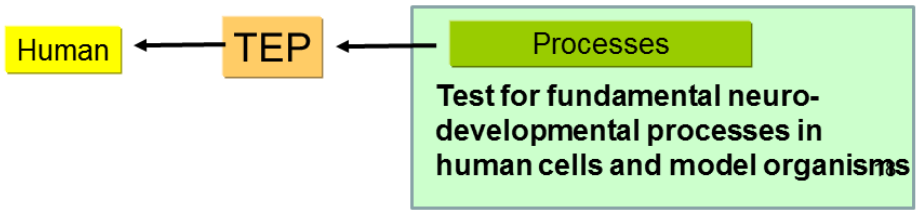


TEP: toxicity endophenotype

John Meynard Keynes:

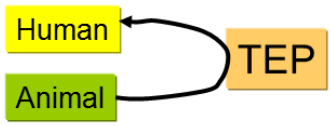
‚The difficulty lies, not in the new *ideas*, but escaping the *old* ones'

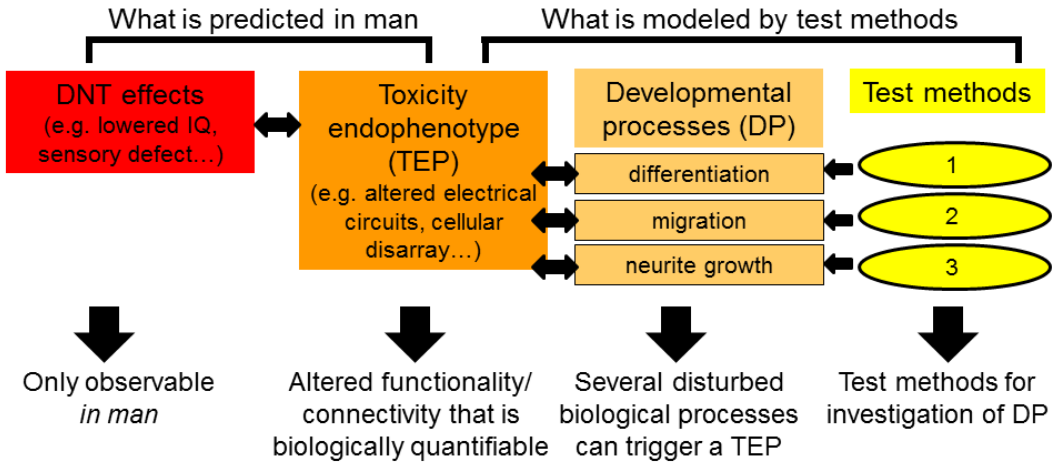
New idea:
Prediction of safety from undisturbed key processes



Old idea:
Prediction of safety from undisturbed phenotype

TEP: toxicity endophenotype





Final conclusion: a process control-based test strategy for DNT

[PPT 11] Introduction to OECD case studies for potential testing strategies and a draft framework for building a DNT testing battery
Ellen Fritsche, Dusseldorf University (DEU)

INTRODUCTION TO OECD CASE STUDIES FOR POTENTIAL TESTING STRATEGIES AND A DRAFT FRAMEWORK FOR BUILDING A DNT TESTING BATTERY

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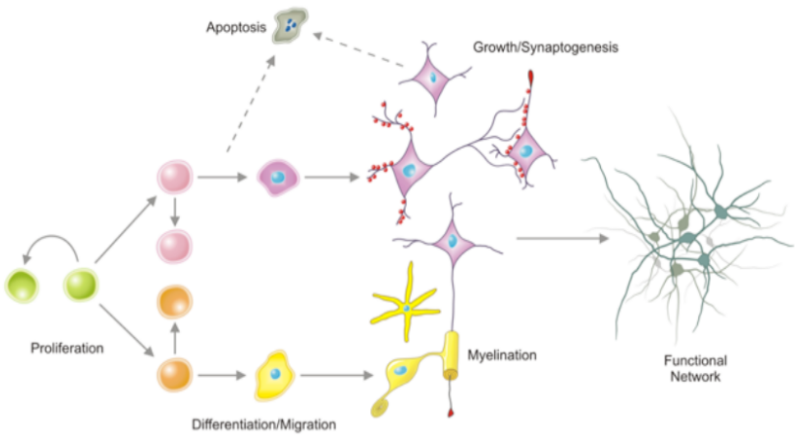
Ellen Fritsche
OECD/EFSA Workshop on DNT: the use of non-animal test methods for regulatory purposes
Brussels, 18-19 October 2016

Mitglied der Leibniz-Gemeinschaft

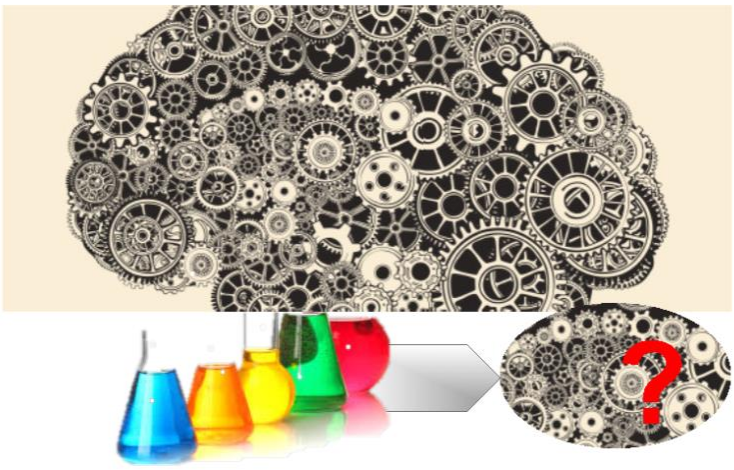
NEURODEVELOPMENTAL PROCESSES SERVING AS POSSIBLE KE FOR DNT TESTING



With courtesy from **William Mundy, U.S. Environmental Protection Agency** and **John Havel, SRA International, Inc.**

IUF LEIBNIZ-INSTITUT FÜR UMWELT-MEDIZINISCHE FORSCHUNG

PROCESS-ORIENTED TESTING STRATEGY



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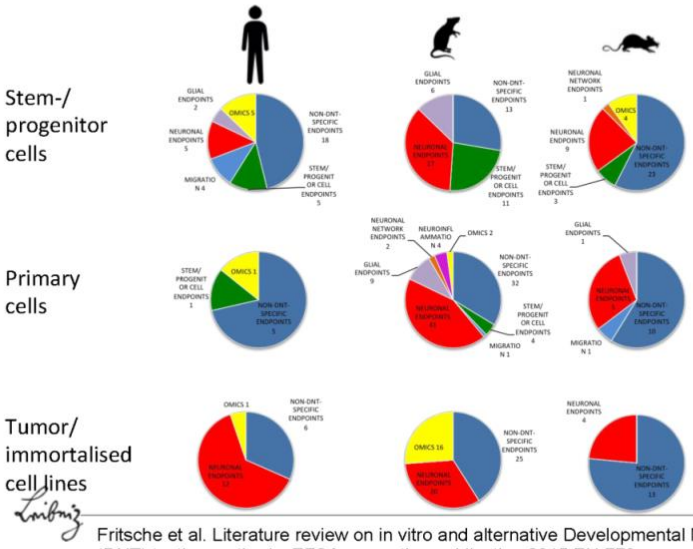
CORRECT CLASSIFICATION OF COMPOUNDS: ENDPOINTS BY CELL TYPES & SPECIES



Fritsche et al. Literature review on in vitro and alternative Developmental Neurotoxicity (DNT) testing methods. EFSA supporting publication 2015:EN-778

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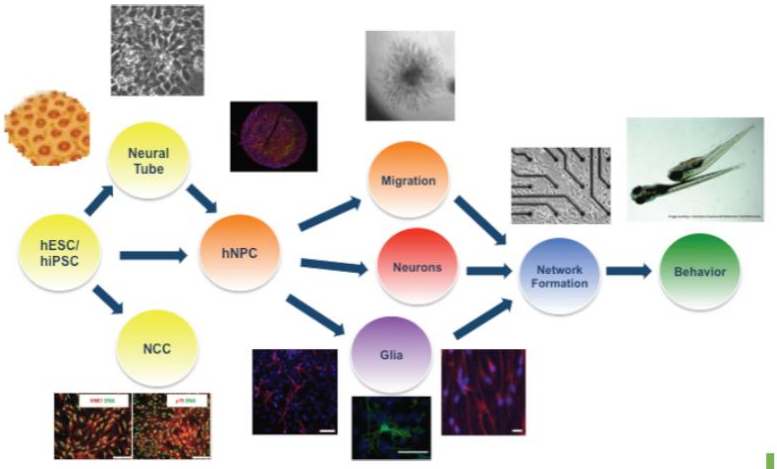
OVERVIEW OVER POTENTIAL DNT TESTING STRATEGY



Fritsche et al. EFSA supporting publication 2015

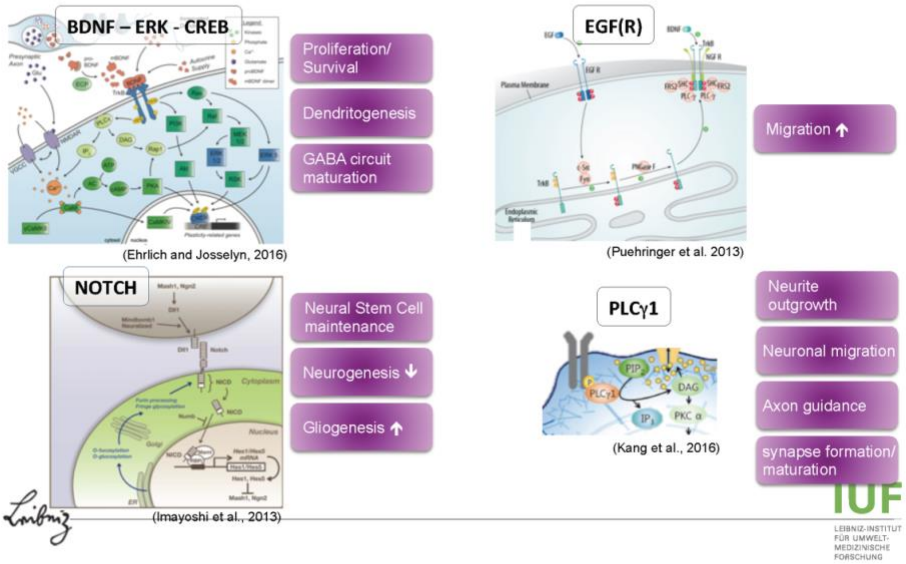
OECD REPORT ON IN VITRO METHODS FOR DNT TESTING

Strategy:

1. Identification of DNT AOPs: 6 (numbers 8, 13, 42, 54, 134, 152; https://aopwiki.org/wiki/index.php/Main_Page)
2. Compound-based MoA evaluation with regards to signaling pathways and neurodevelopmental functions
3. Signaling pathways contributing to human brain development by guiding neurodevelopmental processes

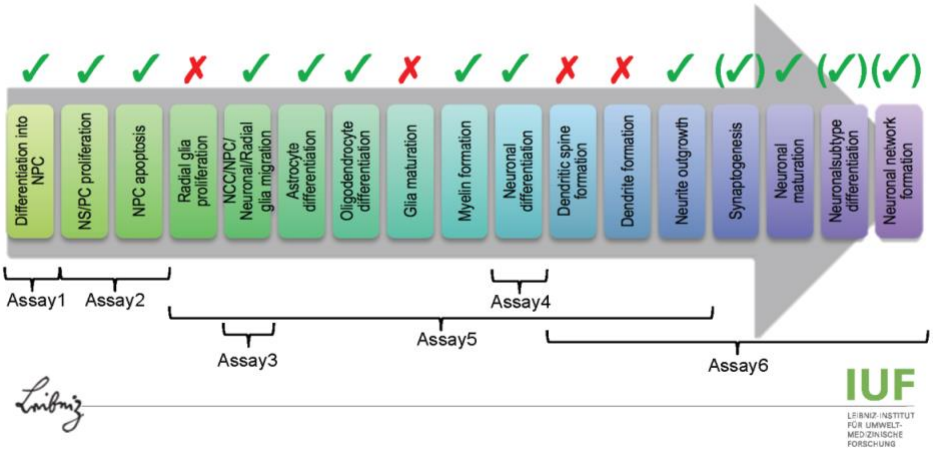
⇨ Extraction of neurodevelopmental processes necessary for brain development: **readiness analyses**

EXAMPLES OF SIGNALING PATHWAYS DRIVING NEURODEVELOPMENTAL PROCESSES



NEURODEVELOPMENTAL PROCESSES INVOLVED IN BRAIN DEVELOPMENT

Availability of HUMAN in vitro method(s) for compound testing on neurodevelopmental endpoints:



DNT ASSAYS FOR HAZARD IDENTIFICATION ON NEURODEVELOPMENTAL ENDPOINTS

Proposed In vitro testing battery:

Assay 1: NPC differentiation

Assay 2: NPC proliferation & apoptosis

Assay 3: NCC migration

Assay 4: Embryonic phase: neuronal differentiation (hESC/hiPSC)

Assay 5: Fetal phase: radial glia/hNPC migration, neuron/astrocyte/ oligodendrocyte differentiation (hNPC)

Assay 6: Neuronal maturation (i.e. neurite outgrowth)/synaptogenesis/neuronal network formation (hNS/PC-based method, preferably with neurons and glia present)

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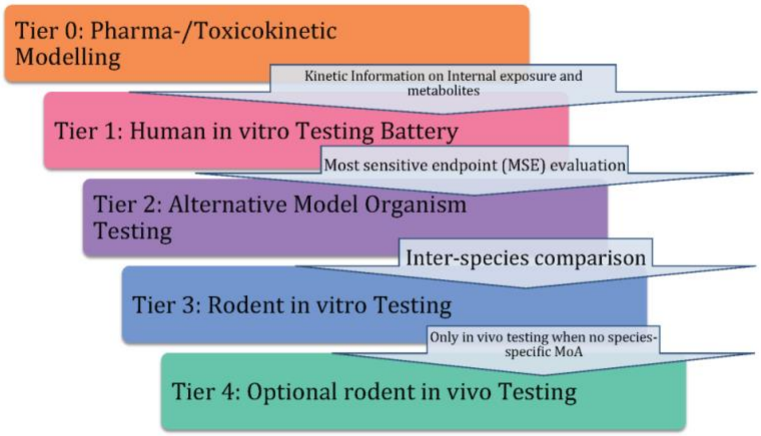
ITS FOR DNT HAZARD IDENTIFICATION: TIERED TESTING STRATEGY



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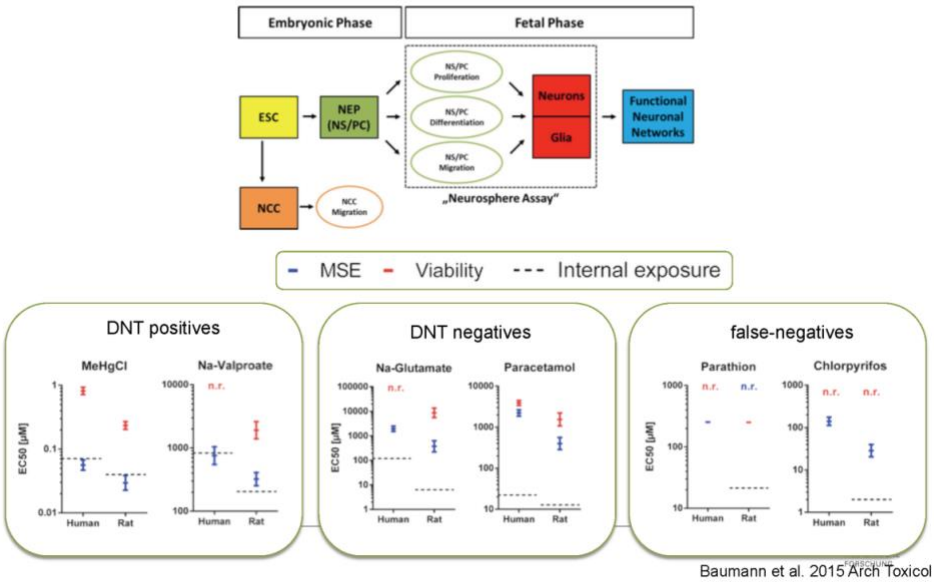
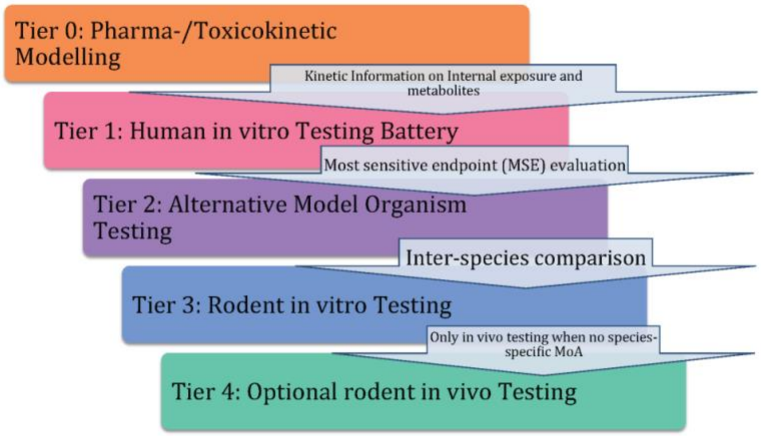
IDENTIFICATION OF MOST SENSITIVE ENDPOINT (MSE) ACROSS TESTING BATTERY



Baumann et al. 2015 Arch Toxicol

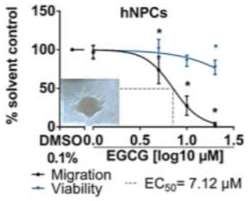
ITS FOR DNT HAZARD IDENTIFICATION: TIERED TESTING STRATEGY



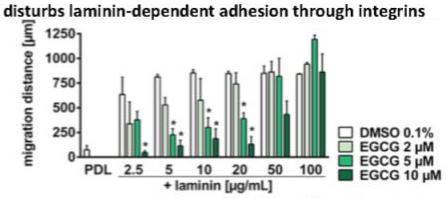
EXAMPLE: FOOD SUPPLEMENT EGCG AS AN UNKNOWN COMPOUND

From **Pharmacokinetics** in Humans and Rats it is estimated that a 3g EGCG/d intake (two tablespoons of commercially available food supplement) of a pregnant woman might lead to a **1 to 3 µM fetal brain concentration**

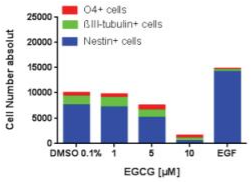
EGCG disturbs hNPC migration



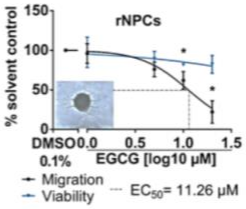
EGCG disturbs laminin-dependent adhesion through integrins



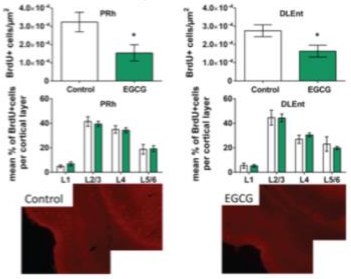
....causing reduction of radial



EGCG disturbs rNPC migration



....and a reduction of BrdU+ cells in P28 cortical layers



there is a concern of high dose food supplement intake during pregnancy. More studies are needed.

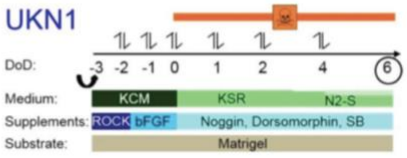
CASE STUDIY: MEHGCL

- Methylmercury causes adverse neurodevelopmental outcomes in children
- It is one of the most data-rich DNT compound
- Data for MeHgCl produced with the proposed assays of the in vitro testing strategy will be displayed

MeHgCl – hESC differentiation into NEP (Assay1)

hNEP differentiation, MeHgCl treatment for 12 days

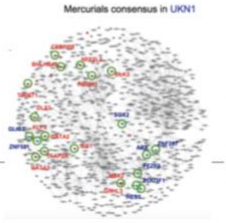
hESC → hNEP



- Cytotoxicity: NOAEC 25 nM
- Functional readout: -
- Gene expression changes: suppressed NCAM1, NEUROD1 and MAP2 expression.

Stummann et al. 2009 Toxicology

- Cytotoxicity: Benchmark concentration (10): 1,5 µM
- Functional readout: -
- Gene expression changes:



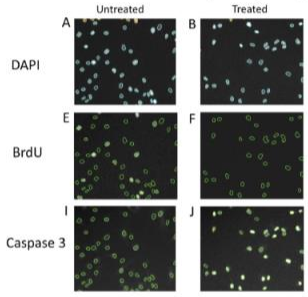
General toxicity factors: Affected by HDACis and mercurials in UKN1

TWIST1	CEBPPB	NFE2L2
PRDM1	ELK3	SIX1
TFAP2A	GATA3	GLIS3
KLF5	GATA2	DLX3
SOX2	ARX	FEZF2
MSX2	GRHL3	POU3F1
HES5	ZNF521	BHLHE40
ZNF287		

Krug et al. 2013,
Shinde et al. 2016 Arch Toxicol

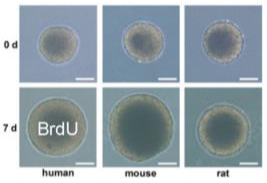
MeHgCl – NPC Proliferation & Apoptosis (Assay2)

ReN CX cells (Millipore)



24 hrs

hNPC (Lonza)



BrdU
72 hrs

- Cytotoxicity >50%: >10 µM
- BrdU incorp. <50%: 30 µM
- Caspase-3 act. >2-fold: -

Culbreth et al. 2012 NeuroToxicol

- Cytotoxicity >50%: 1,100 nM
- BrdU incorp. <50%: 700 nM
- Caspase-3 act. >2-fold: n. d.

Baumann et al. 2016, Arch Toxicol

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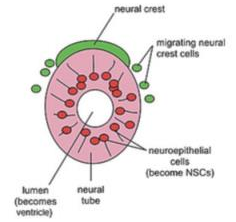
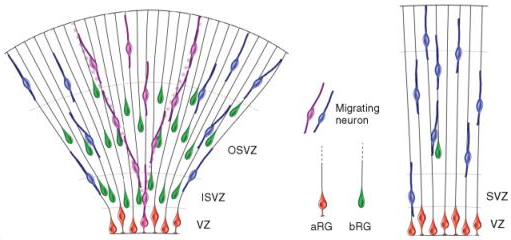
MIGRATION DURING BRAIN DEVELOPMENT

NCC/NEP migration



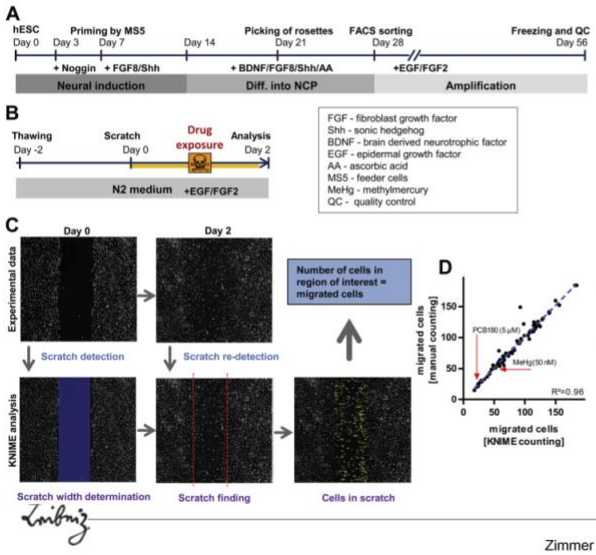
http://www.glycoforum.gr.jp/science/hyaluronan/HA30/HA30E.html

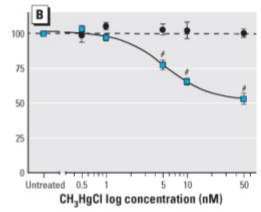
Radial glia cell/Neuronal migration



Borrell & Götz 2014 Curr Opin Neurobiol

MEHGCL – NEURAL CREST CELL MIGRATION (MINC) ASSAY (HESC-BASED, ASSAY 3)



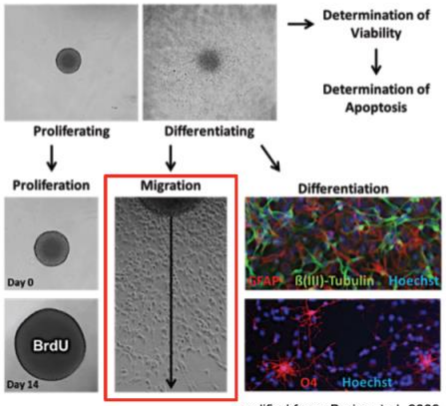


- Cytotoxicity >50%: n.r.
- Migration <50%: n.r., significant red.: 5 nM

Zimmer et al., 2012 Environ Health Perspect

MIGRATION ASSAYS (II) – NPC MIGRATION ASSAY (PART OF ASSAY 5)

The 'Neurosphere Assay' (hNPC, Lonza)



modified from: Breier et al. 2009

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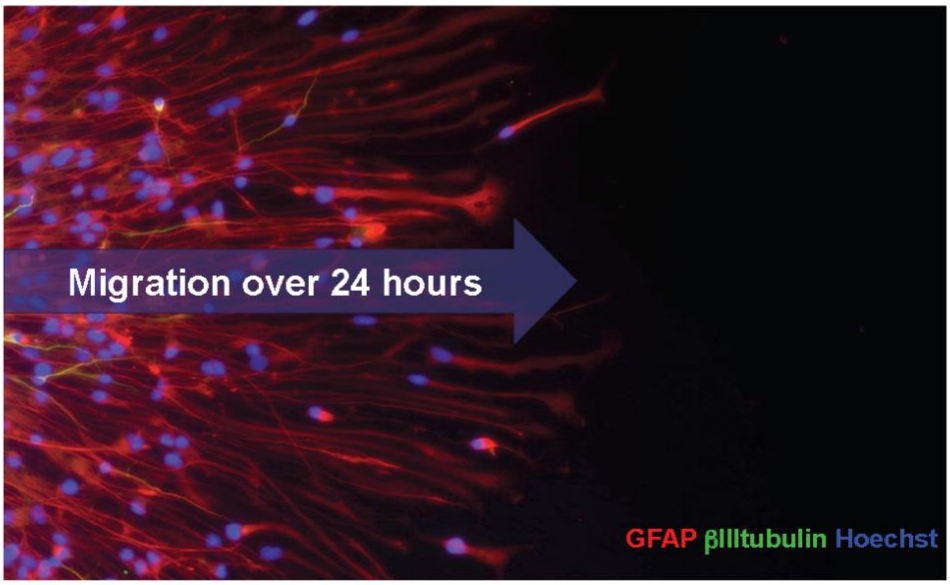
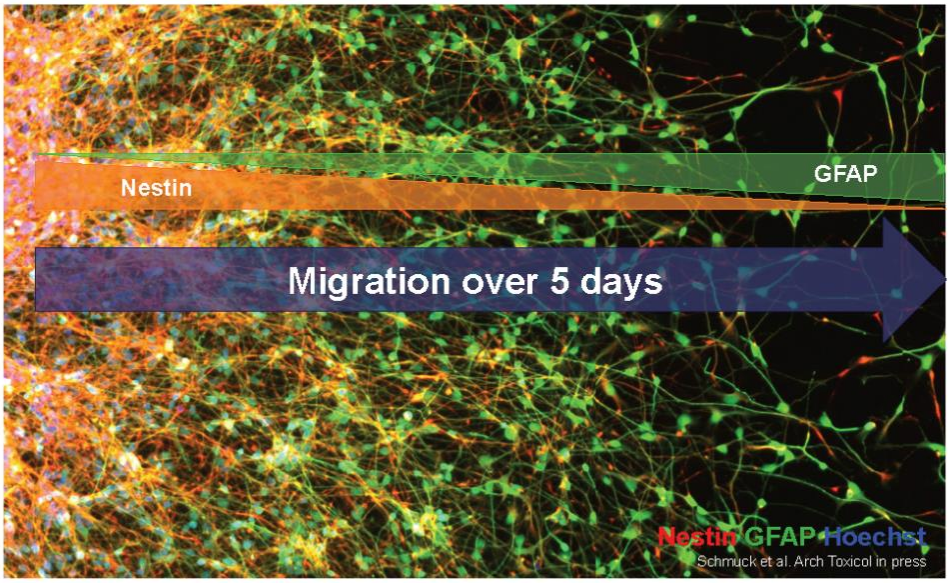
NPC MIGRATION ASSAY – RADIAL GLIA MIGRATION

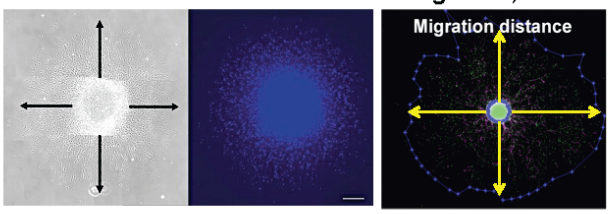


MIGRATION ASSAYS (II) – NPC MIGRATION ASSAY – RADIAL GLIA MIGRATION



MIGRATION ASSAYS (II) – NPC MIGRATION ASSAY (PART OF ASSAY 5)

Radial Glia Migration, 24 hrs

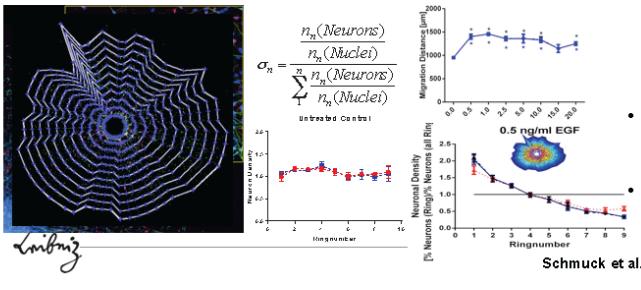


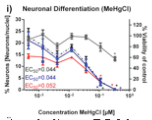
Cytotoxicity >50%: >3 µM

Migration <50%: 650 nM, significant red.: 30 nM

Moors et al. 2007, Baumann et al. 2015,

Neuronal migration (5 days)



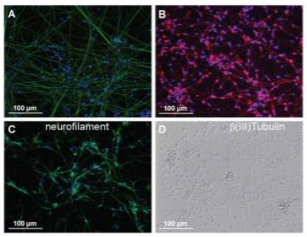


- Cytotoxicity >50%: n.r., significant red.: 330 nM (40%)
- Neuronal migration <50%: -

Schmuck et al. Arch Toxicol in press

NEURONAL DIFFERENTIATION

hESC-based (Assay 4)



Stummann et al. 2009 Toxicology

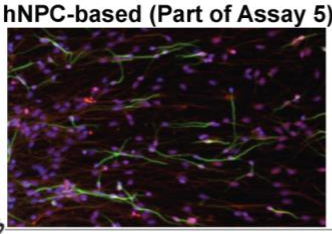
After 12 days:
- Cytotoxicity >50%: 39 nM
- Neuronal Differentiation (Map2/Ncam/ NeuroD gene expression) at NOAEC: 25 nM

Stummann et al. 2009, Toxicology

After 13 days:
- Cytotoxicity >50%: 100 nM
- Neuronal Differentiation (nº Map2+ cells) <50%: ≥ 1 nM

He et al. 2012, Tox Lett

hNPC-based (Part of Assay 5)



After 72 hrs:
- Cytotoxicity >50%: 800 nM
- Neuronal Differentiation <50%: 60 nM
- significant red.: 12 nM

Baumann et al. 2016, Arch Toxicol

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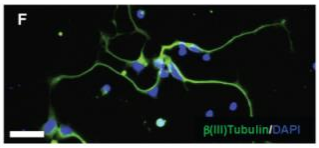
NEURITE OUTGROWTH ASSAYS

hESC/hNPC-based methods:

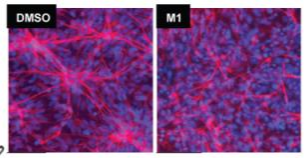
- hN2^{TM} (Aruna, Assay 6): single cell type (neuronal) cultures, HCA



After 24 hrs:
- Cytotoxicity (neurons/field) <75%: 90 nM
- Neurite Count <75%: 70 nM
- Neurite Length <75%: 200 nM

(Harrill et al. 2011 TAAP)

- He et al. (2012) uses hESC-derived neural mixed-cultures, HCA (Assay 4)



After 13 days:
- Cytotoxicity (MTT assay) <75%: 100 nM (stat.) IC_{50} ca. 400 nM
- Neurite Length <60%: 1 nM (stat.)
- Branching points <60%: 100 nM

(He et al. 2012 Tox Lett)

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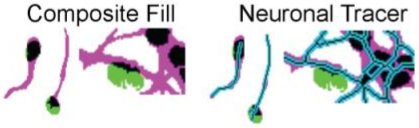
NEURITE OUTGROWTH ASSAY (CTD.)

hESC/hNPC-based methods:

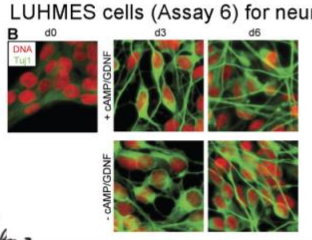
- hNPC-derived young neurons in mixed and mixed-density cultures (Assay 5)



After 5 days:
- Cytotoxicity >50%: n.r., significant red.: 330 nM (40%), neur. diff. <50%: 45 nM
- Neurite Length/neuron <50%: ≈200 nM
- Branching points/neuron <50%: ≈200 nM
- Neurites/neuron <50%: ≈200 nM

(Schmuck et al. in press)

- LUHMES cells (Assay 6) for neurite outgrowth assessment (Stiegler et al. 2011, Krug et al. 201



After 24 hrs:
- Cytotoxicity (neurons/field) <50%: 110 nM
- Neurite Area <50%: 90 nM

(Stiegler et al. 2011 Tox Sci)

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SENSITIVITY OF IN VITRO ASSAYS TOWARDS MEHGCL: SHORT-TERM ASSAYS UP TO 72 HRS

IC_{25} & statistical significance

Assay 3	Assay 5	Assay 5	Assay 6a	Assay 6b	Assay 6b	Assay 2a	Assay 2b
NCC migration 24 h	hNPC radial glia migration 24 h	hNPC (neurosphere) neuron. diff. 3 d	LUHMES neurite area 24 h	hN2 neurite count 24 h	hN2 neurite length 24 h	hNPC BrdU 3 d	ReN CX BrdU 24 h
5 nM	30 nM	37 nM	40 nM	70 nM	200 nM	700 nM	3,000 nM
>50 nM stat.	≥3000 nM stat.	(12 nM) stat.	100 nM stat.	90 nM IC_{25}	90 nM IC_{25}	800 nM stat.	3,000 nM stat.

IC_{50}	Assay 5	Assay 6a	Assay 5	Assay 2a	Assay 2b			
	hNPC (neurosphere) neuron. diff. 3 d	LUHMES neurite area 24 h	hNPC radial glia migration 24 h	hNPC BrdU 3 d	ReN CX BrdU 24 h	hN2 neurite count	hN2 neurite length	NCC migration
	60 nM	90 nM	650 nM	700 nM	30,000 nM			
	800 nM	110 nM	n.r.	1100 nM	>10,000 nM			

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SENSITIVITY OF IN VITRO ASSAYS TOWARDS MEHGCL: LONG-TERM ASSAYS 5 TO 13 DAYS

IC_{25}, NOAEC, BMC & statistical significance

	Assay 6c	Assay 4	Assay 5	Assay 6c	Assay 5	Assay 1	Assay 5
	hESC-der. mixed cultures neurite length 13 d	hESC neuron. diff. gene expression 12 d	hNPC (Neurospheres) neuronal diff. 5 d	hESC-der. mixed cultures branching points 13 d	hNPC (Neurospheres) neurite outgrowth 5 d	UKN1 hESC-NEP diff. gene expression 6 d	hNPC (Neurospheres) neuron migration 5 d
	1 nM	25 nM	100 nM	100 nM	200 nM	1500 nM	- nM
	100 nM stat.	25 nM NOAEC	330 nM stat.	100 nM stat.	330 nM stat.	1500 nM BMC	330 nM stat.

IC_{50}	Assay 4	Assay 5	Assay 5	Assay 6c	Assay 4
	hESC-der. mixed cultures neuron. diff 13 d	hNPC (Neurospheres) neuronal diff. 5 d	hNPC (Neurospheres) neurite outgrowth 5 d	hESC-der. mixed cultures branching points 13 d	hESC neuron. diff. gene expression 12 d
	<1 nM	44 nM	200 nM	100 nM	190 nM
	400 nM	n.r.	n.r.	100 nM	190 nM

MEHGCL: IN VIVO – IN VITRO EXTRAPOLATION APPROACH

Maternal hair concentration of 4.5 ppm (LOAEL) ⇨ Neuropsychological deficits in children (Castoldi et al. 2001)

⇩ (Burbacher et al. 1990; Lewandowski et al. 2003)

approx. 72 nM ⇨

- Migration
- Neuronal Differentiation
- Neuronal Maturation (Neurite Outgrowth)

Burbacher TM, Rodier PM, Weiss B (1990) Methylmercury developmental neurotoxicity: a comparison of effects in humans and animals. Neurotoxicol Teratol 12(3):191–202
Castoldi AF, Coccini T, Ceccatelli S, Manzo L (2001) Neurotoxic- ity and molecular effects of methylmercury. Brain Res Bull 55(2):197–203
Lewandowski T, Ponce R, Charleston J, Hong S, Faustman E (2003) Effect of methylmercury on midbrain cell proliferation during organogenesis: potential cross-species differences and implica- tions for risk assessment. Toxicol Sci 75(1):124–133

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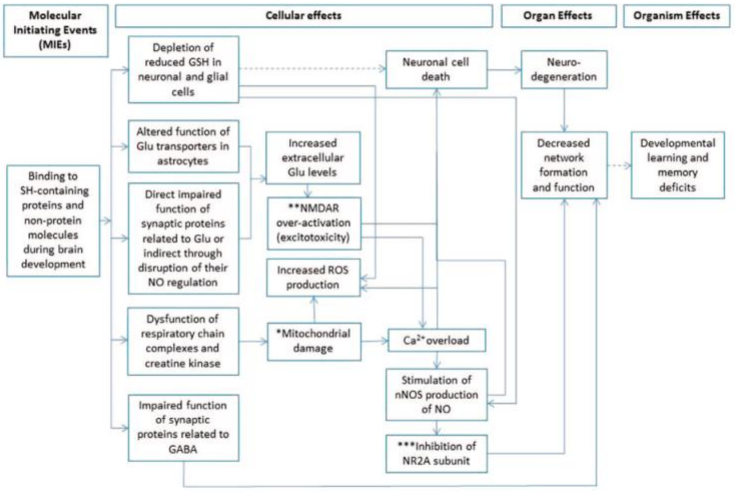
MeHgCl: putative AOP on chemicals binding to SH-groups

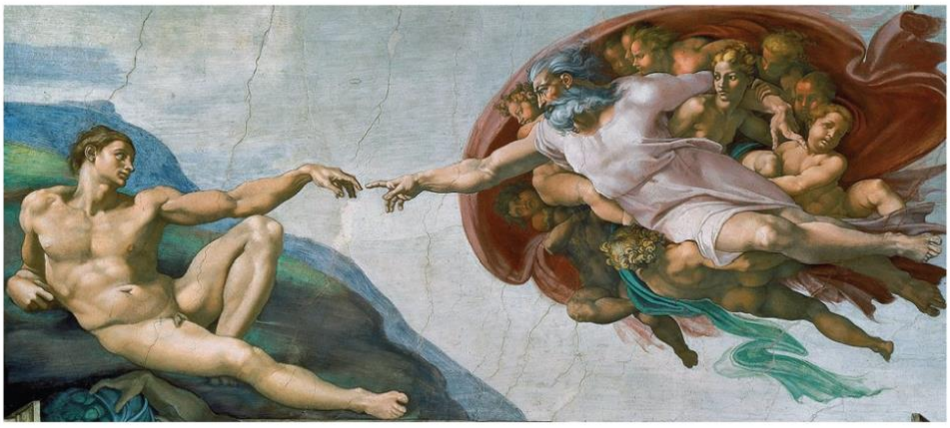


Bal-Price et al. 2015 Crit Rev Toxicol; AOP VI

Summary & Conclusion

- From the current state of science, DNT in vitro testing can be based on neurodevelopmentally-relevant processes that can serve as KE in an AOP-based framework
- 'Signaling pathway' to 'process function' analyses improve confidence in assays, which is necessary for regulatory acceptance
- A large variety of neural stem/progenitor cell-based DNT in vitro assays is available NOW
- Compound testing across a battery of in vitro tests covering timing and processes of brain development is the next step forward

SUMMARY & CONCLUSION



ACKNOWLEDGEMENTS

- OECD funded Report on 'Report on Integrated Testing Strategies for the identification and evaluation of chemical hazards associated with the developmental neurotoxicity (DNT), to facilitate discussions at the Joint EFSA/ OECD Workshop on DNT', October 18th/19th, Brussels

- EFSA funded 'Literature review on in vitro and alternative Developmental Neurotoxicity (DNT) testing methods' (2015)

CASE STUDIES: MEHGCL SUMMARY OF IN VITRO HAZARD

Endpoint	In vitro system	Assay number	Time of Exposure	Measure	Effective concentration Endpoint	Measure	Effective concentration Viability
NEP differentiation (MAP2 gene expression)	hESC	1	12 Days	Endpoint determination	25 nM	NOAEC	25 nM
NEP differentiation (global gene expression)	hESC	1	6 days	Endpoint determination	1000 nM	Benchmark concentration (10)	1000 nM
NPC apoptosis (Cspase-3 activation)	ReN CX cells (Millipore)	2	24 hrs	2-fold induction	-	IC_{50}	-
NPC proliferation (BrdU incorp.)	ReN CX cells (Millipore)	2	24 hrs	IC_{50}	30,000 nM	IC_{50}	-
NPC proliferation (BrdU incorp.)	hNPC (Neurospheres)	2	72 hrs	IC_{50}	700 nM	IC_{50}	1,100 nM
Neural crest cell migration (MINC Assay)	hESC-der NCC	3	48 hrs	IC_{50} Stat. signif.	n.r. 50 nM	IC_{50}	n.r., >50 nM
Radial glia migration assay	hNPC (Neurospheres)	4	24 hrs	IC_{50} Stat. signif.	650 nM 30 nM	IC_{50}	n.r., ≥3000 nM

SENSITIVITY OF IN VITRO ASSAYS TOWARDS MEHGCL: SHORT-TERM ASSAYS UP TO 72 HRS

Cell Endpoint	Cultures	Exposure time	Measure	Effect. Conc. Endpoint	Measure	Effect. Conc. Viability	Literature
ReN CX cell BrdU	Myc-immort. NPC	24 hrs	IC_{50} Stat.	30,000 nM 3,000 nM	IC_{50} Stat.	- 3,000 nM	Culbreth et al. 2012 Breier et al. 2008
Radial glia migration	Primary NPC Neurosphere Assay	24 hrs	IC_{50} Stat.	650 nM 30 nM	IC_{50} Stat. signif.	n.r. ≥3000 nM	Baumann et al. 2015
Neurite Count	hN2	24 hrs	IC_{25}	70 nM	IC_{25}	90 nM	Harrill et al. 2011
Neurite Length	hN2	24 hrs	IC_{25}	200 nM	IC_{25}	90 nM	Harrill et al. 2011
Neurite Area	LUHMES	24 hrs	IC50 Stat. signif.	90 nM 40 nM	IC50 Stat. signif.	110 nM 100 nM	Stiegler et al. 2011
NCC migration	MINC Assay hESC-NCC	48 hrs	IC_{50} Stat.	n.r. 50 nM	IC_{50} Stat.	n.r. >50 nM	Zimmer et al., 2012
hNPC BrdU	Primary NPC Neurosphere Assay	72 hrs	IC_{50}	700 nM	IC_{50}	1,100 nM	Baumann et al. 2015
hNPC neuronal diff	Primary NPC Neurosphere Assay	72 hrs	IC_{50} Stat. signif.	60 nM 37 nM (>40% reduction)	IC_{50}	800nM 12 nM (<25% reduction 12 and 37 nM)	Baumann et al. 2015

SENSITIVITY OF IN VITRO ASSAYS TOWARDS MEHGCL

- **Long-term Assays 5 to 13 days**

Cell Endpoint	Cultures	Exposure time	Measure	Effect. Conc. Endpoint	Measure	Effect. Conc. Viability	Literature
Neuronal differentiation	hNPC (Neurospheres)	5 days	IC_{50} Stat. sign.	44 nM 100 nM	IC_{50} Stat. signif.	n.r. 330 nM (40% reduction)	Schmuck et al. 2016
Neurite outgrowth assay Neurite Count	hNPC (Neurospheres)	5 days	IC_{50} Stat. signif.	200 nM	IC_{50} Stat. signif.	n.r. 330 nM (40% reduction)	Schmuck et al. 2016
Neurite outgrowth assay Neurite Length	hNPC (Neurospheres)	5 days	IC_{50} Stat. signif.	200 nM	IC_{50} Stat. signif.	n.r. 330 nM (40% reduction)	Schmuck et al. 2016
Neurite outgrowth assay Neurite Branching points	hNPC (Neurospheres)	5 days	IC_{50} Stat. signif.	200 nM	IC_{50} Stat. signif.	n.r. 330 nM (40% reduction)	Schmuck et al. 2016
Neuron migration assay	hNPC (Neurospheres)	5 days	IC_{50} Stat. signif.	-	IC_{50} Stat. signif.	n.r. 330 nM (40% reduction)	Schmuck et al. 2016
NEP differentiation (global gene expression), UKN1	hESC	6 days	Endpoint determination	1500 nM	Benchmark concentration (10)	1500 nM	Shinde et al. 2016

SENSITIVITY OF IN VITRO ASSAYS TOWARDS MEHGCL

- **Long-term Assays 5 to 13 days (cont.)**

Cell Endpoint	Cultures	Exposure time	Measure	Effect. Conc. Endpoint	Measure	Effect. Conc. Viability	Literature
NEP differentiation (Map2 gene expression)	hESC	12 days	Endpoint determination	25 nM	NOAEC	25 nM	Stummann et al. 2009
Neuronal differentiation assay (Map2 gene expr)	hESC	12 days	IC_{50}	190 nM	IC_{50}	190 nM	Stummann et al. 2009
Neuronal differentiation assay (Map2+ cells)	hESC-der. mixed cultures	13 days	IC_{50}	1 nM	IC_{50} Stat. sign.	ca. 400 nM 100 nM	He et al. 2012
Neurite outgrowth assay Neurite Length	hESC-der. mixed cultures	13 days	Stat. sign. <60%	1 nM	IC_{50} Stat. sign.	ca. 400 nM 100 nM	He et al. 2012
Neurite outgrowth assay Neurite Branching points	hESC-der. mixed cultures	13 days	Stat. sign.	100 nM	IC_{50} Stat. sign.	ca. 400 nM 100 nM	He et al. 2012

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