Alternative Test Methods for Developmental Neurotoxicity: A History and Path Forward

Kevin M. Crofton
Deputy Director
National Center for Computational Toxicology

OECD/EFSA Workshop
18 October 2016
Brussels

Office of Research and Development
National Center for Computational Toxicology

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

From Makris et al., EHP 2009

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

Problem: Evidence for Increasing Incidence of Neurodevelopmental Disorders

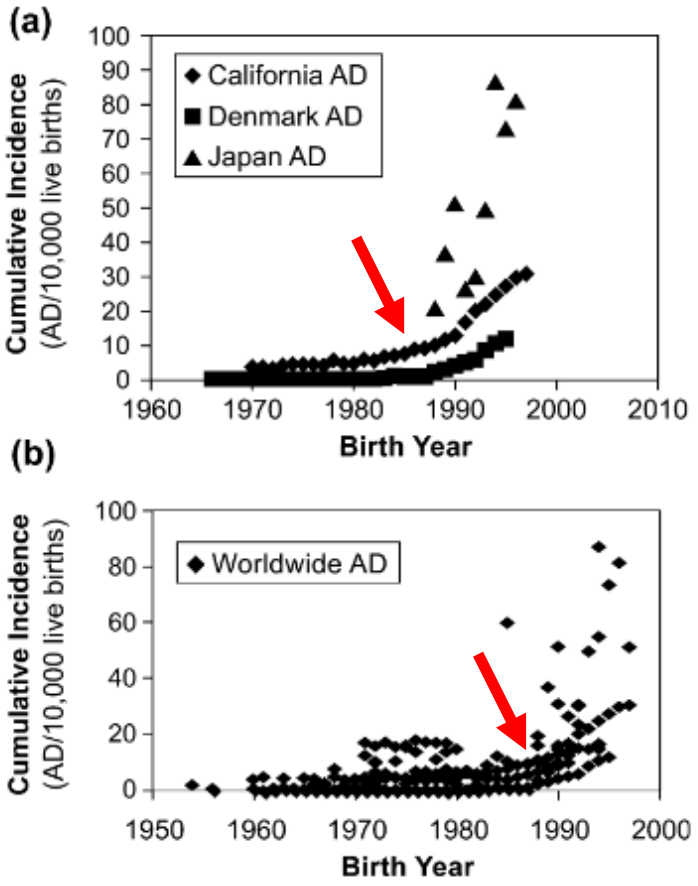
- Prevalence of neurodevelopmental diseases in children increased (Atladottir et al. 2015; Landrigan et al 2012)
- Overall estimates that 10-15% in children (Grandjean & Landrigan, Lancet 2014)
- Genetic factors account for no more than 30–40% (NRC, 2000)
- Includes: autism spectrum, ADHD, dyslexia,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

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

Timing of Increased Autistic Disorder Cumulative Incidence

MICHAEL E. MCDONALD* AND JOHN F. PAUL

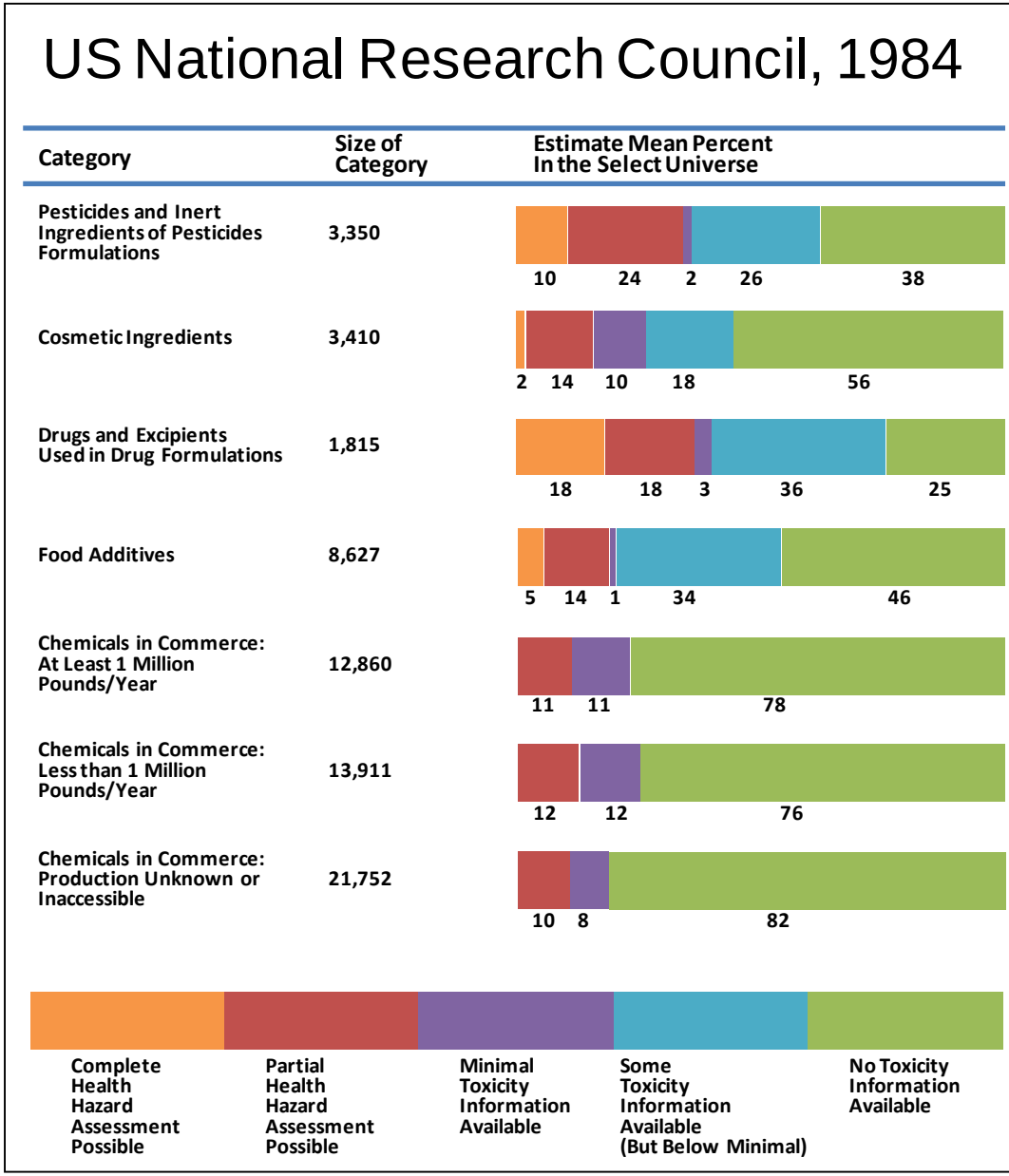
Environ. Sci. Technol. **2010,** 44, 2112–2118



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

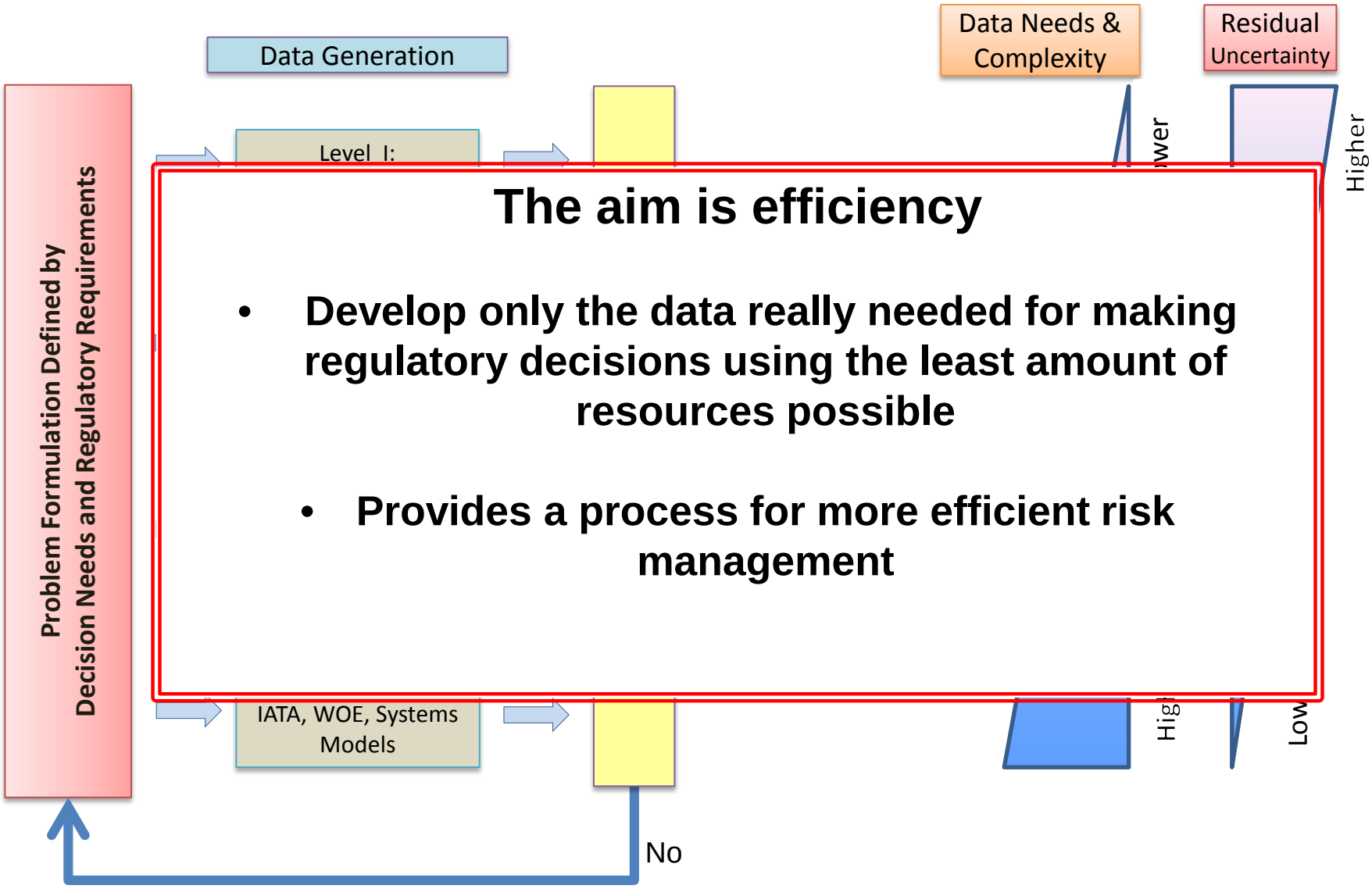
Data Types & Chemical Risk Decisions

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

Matching Data Type and Uncertainties to Decision Context



The aim is efficiency

- **Develop only the data really needed for making regulatory decisions using the least amount of resources possible**
- **Provides a process for more efficient risk management**

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?

Black dot = no DNT study

Red dot = DNT study*

* ~0.2% DNT Guideline Studies

Progress to Date – In Vitro

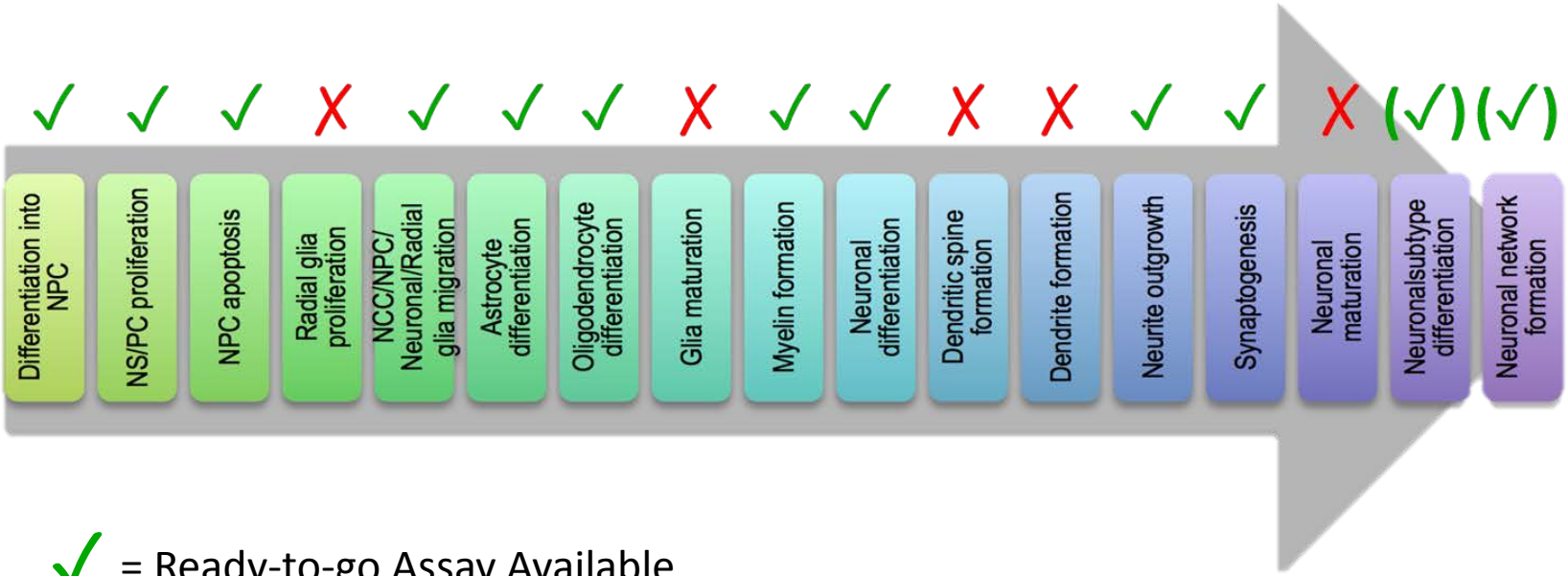
Critical Science Challenges for DNT*

- 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!

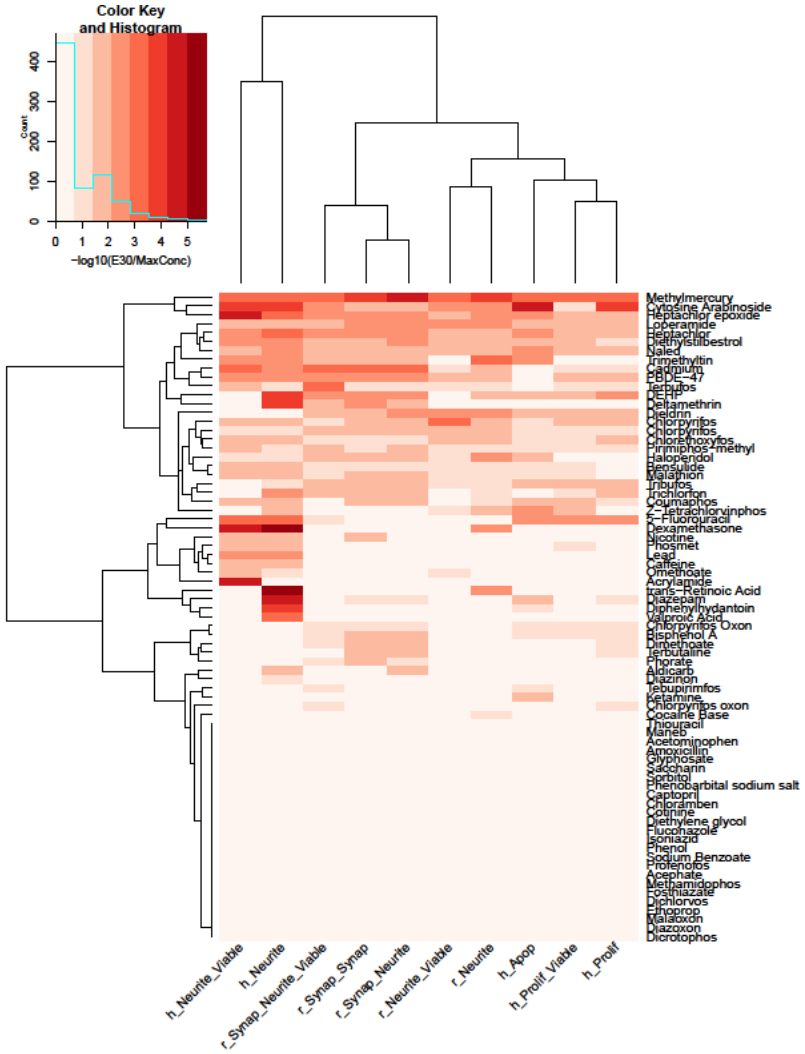
Mundy and colleagues
Hierarchical Clustering of Potency of Multiple DNT Endpoints

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

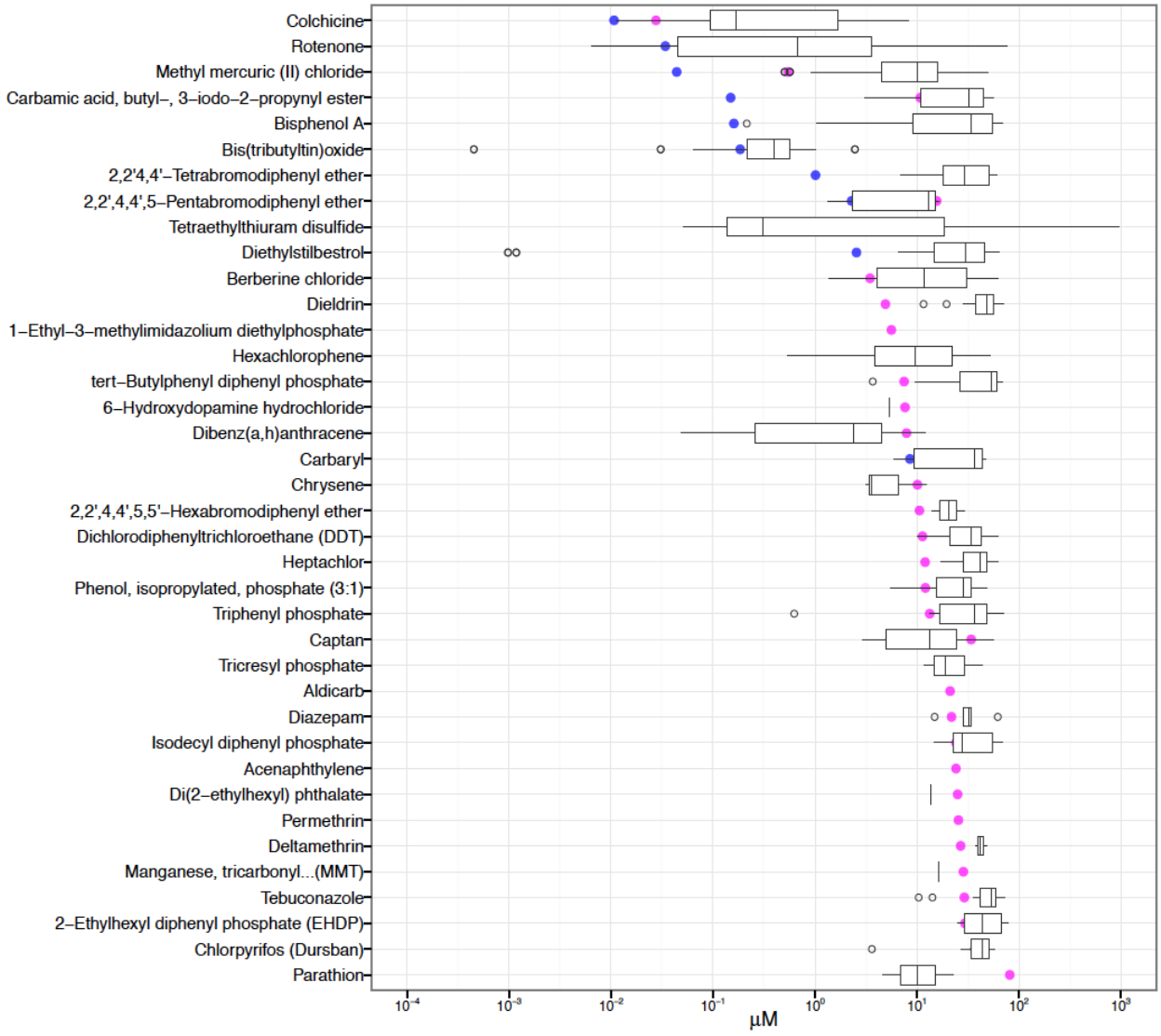


Allows prioritization by:
1. Overall potency
2. Selectivity for neurodevelopment endpoints (not shown)

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

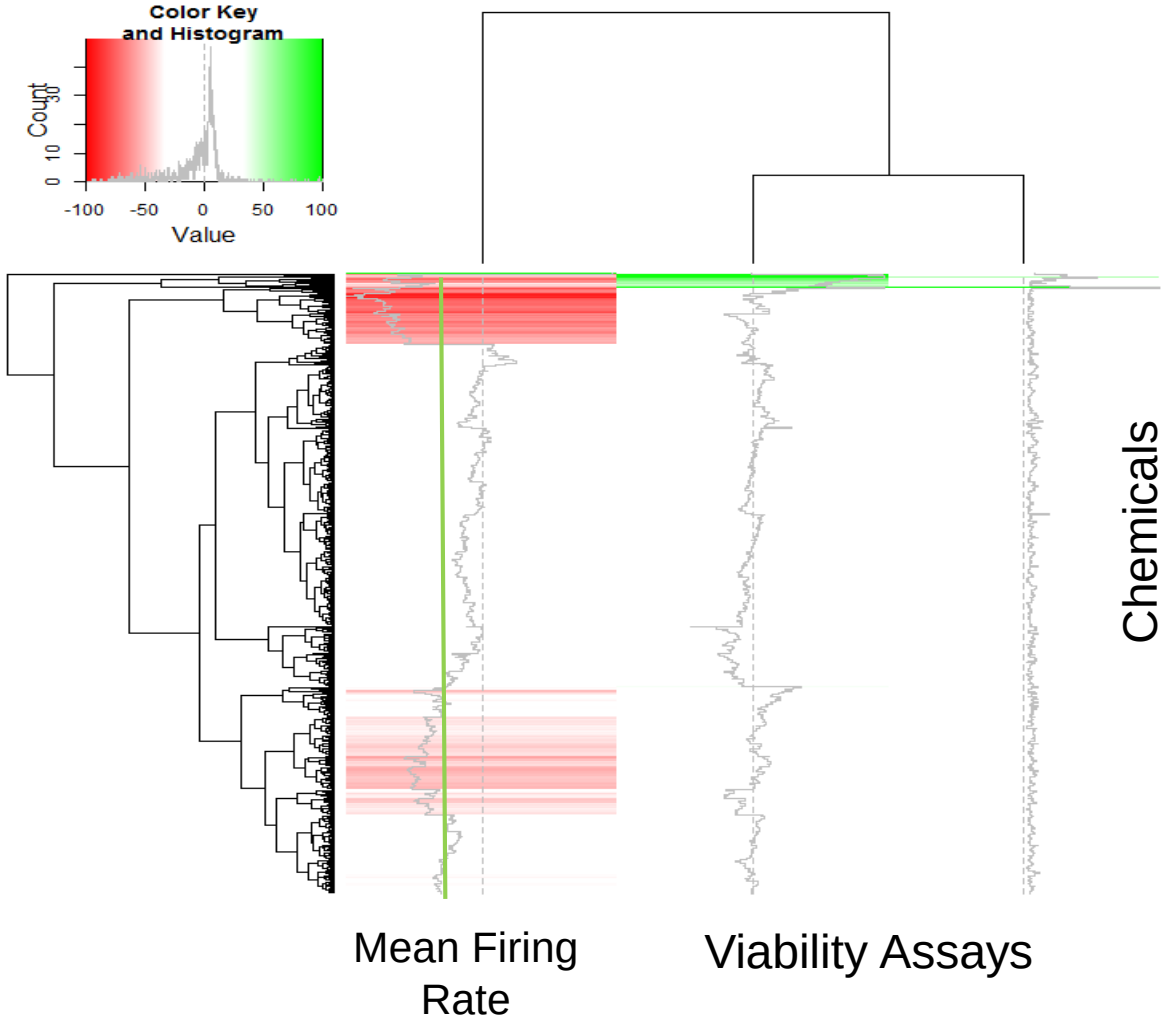
iPSC
LUHMES
Tox21

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

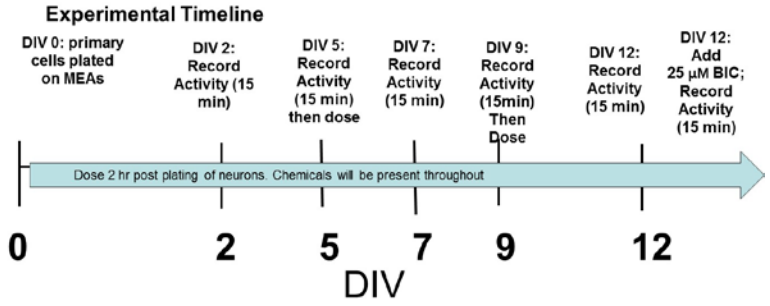


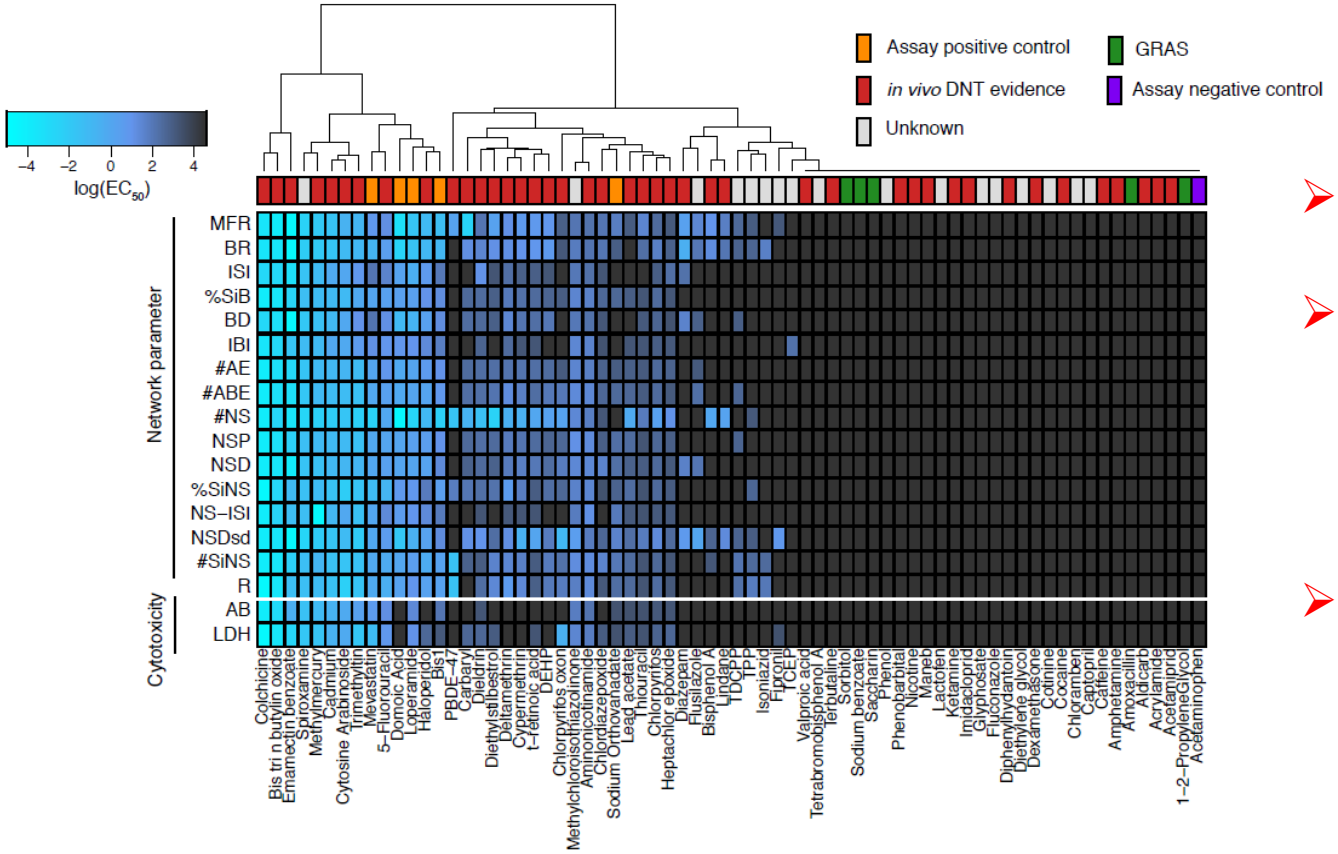
Public release of data release via ToxCastDB in 2017

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?

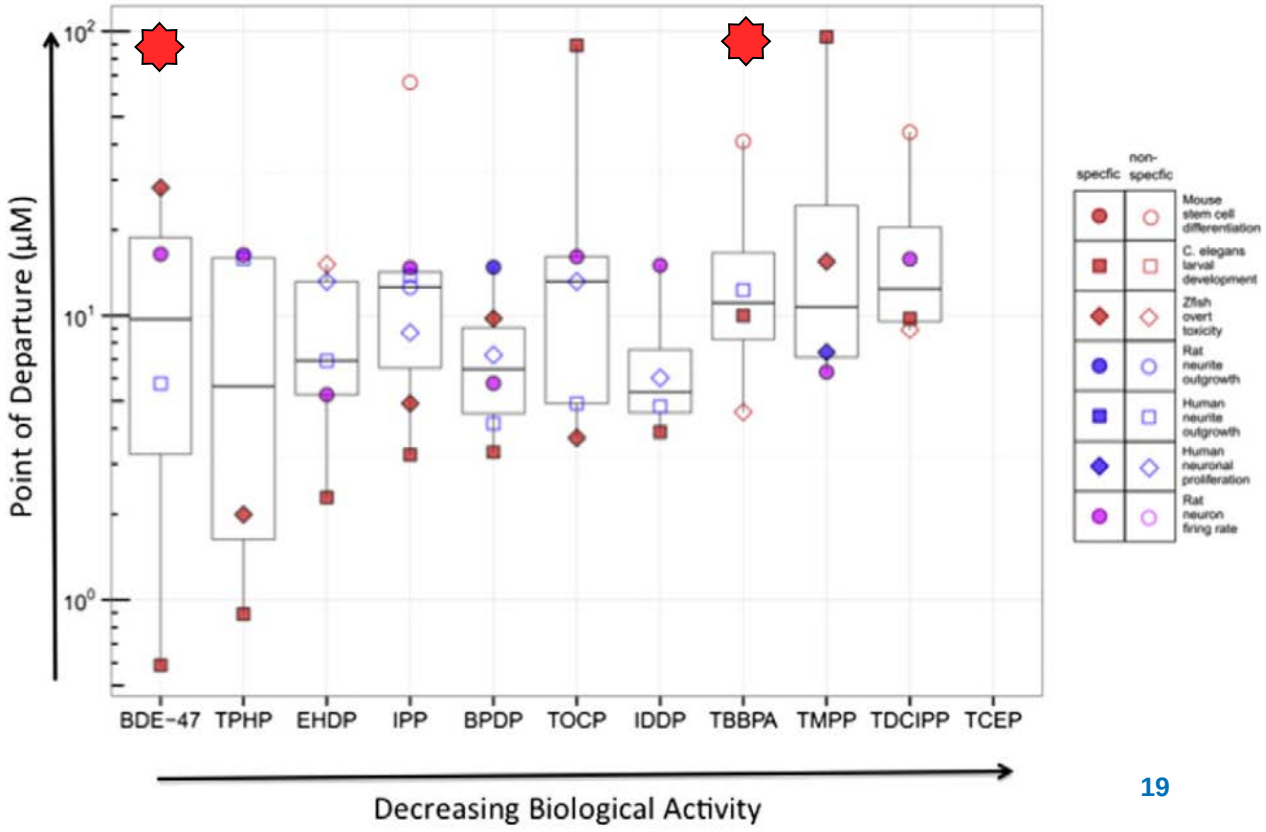
Courtesy of T. Shafer

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

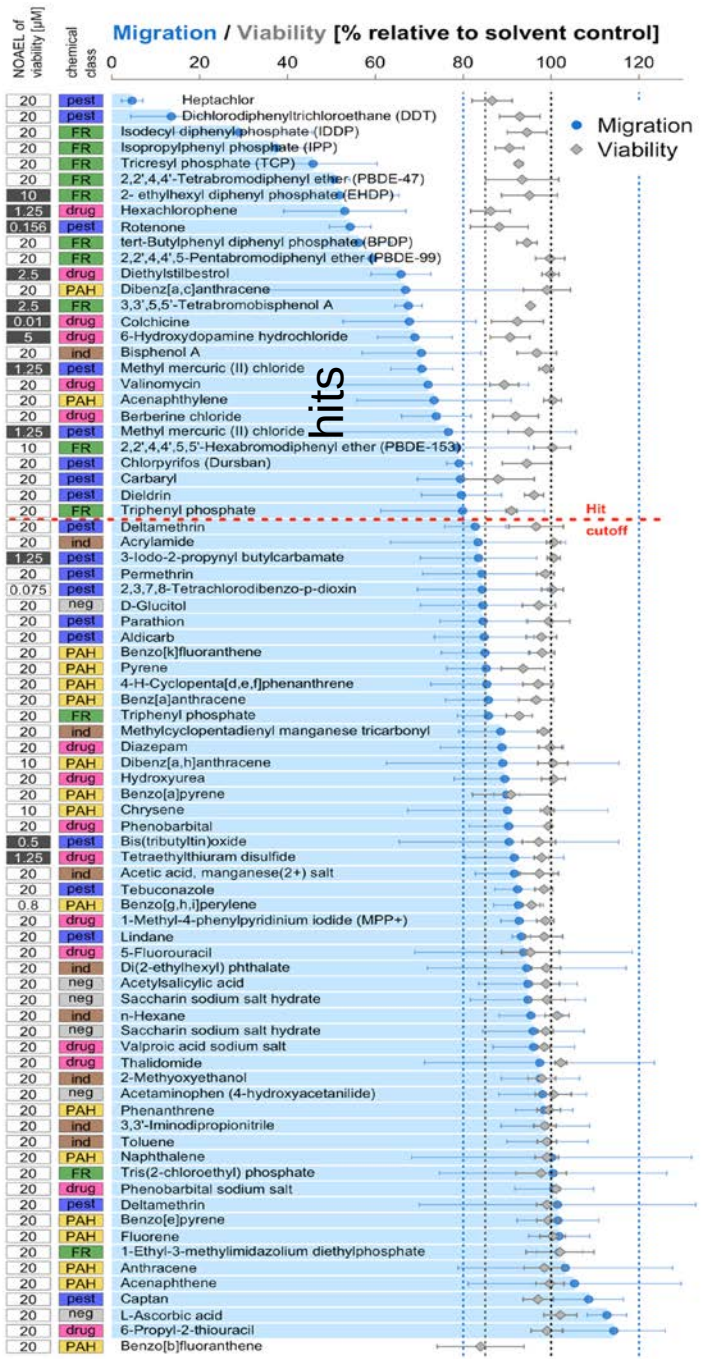
- Purpose: Compare BFRs to replacement OP-FRs via bioactivity
- Use battery approach – in vitro devtox and DNT assays
  - proliferation, viability, neurite outgrowth, MEAs, cytotoxicity, devtox assays
- 11 organophosphate and brominated flame retardants
- Compare in vitro PODs

Conclusions:

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



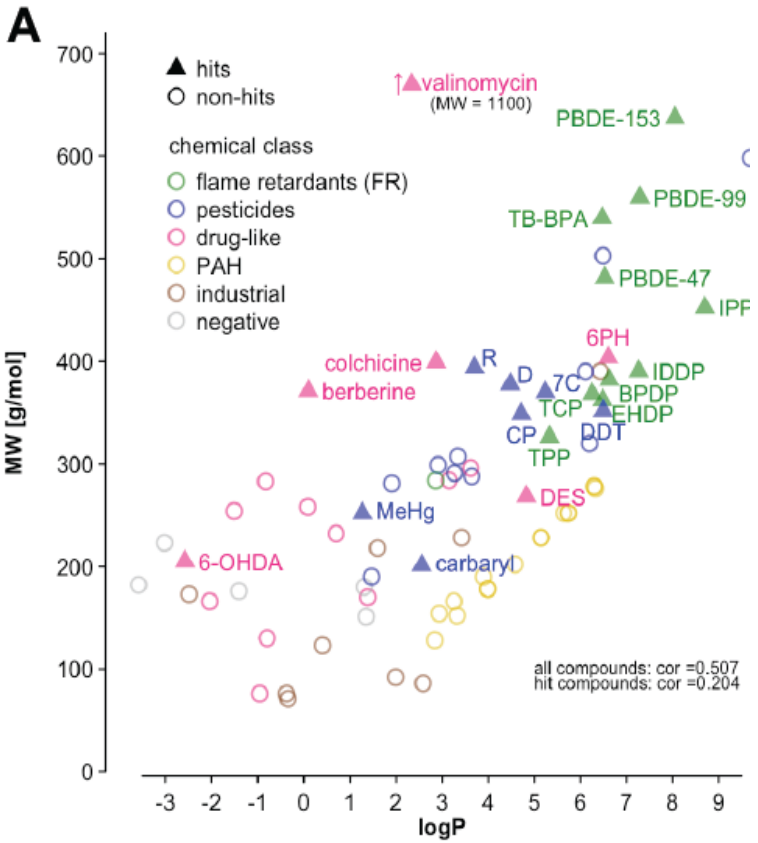
Leist and colleagues
NTP80 and Neural crest assay



(Nyffeler et al., unpublished)

- 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*

logP vs MW chemical space

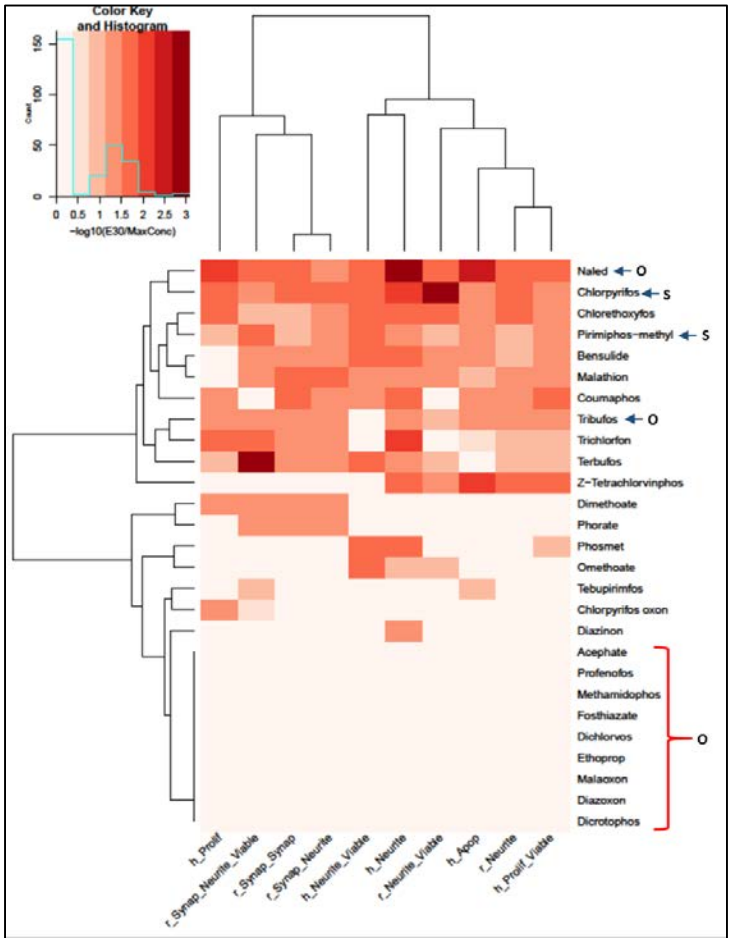


EPA OPP-NHEERL Organophosphate Project

- 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

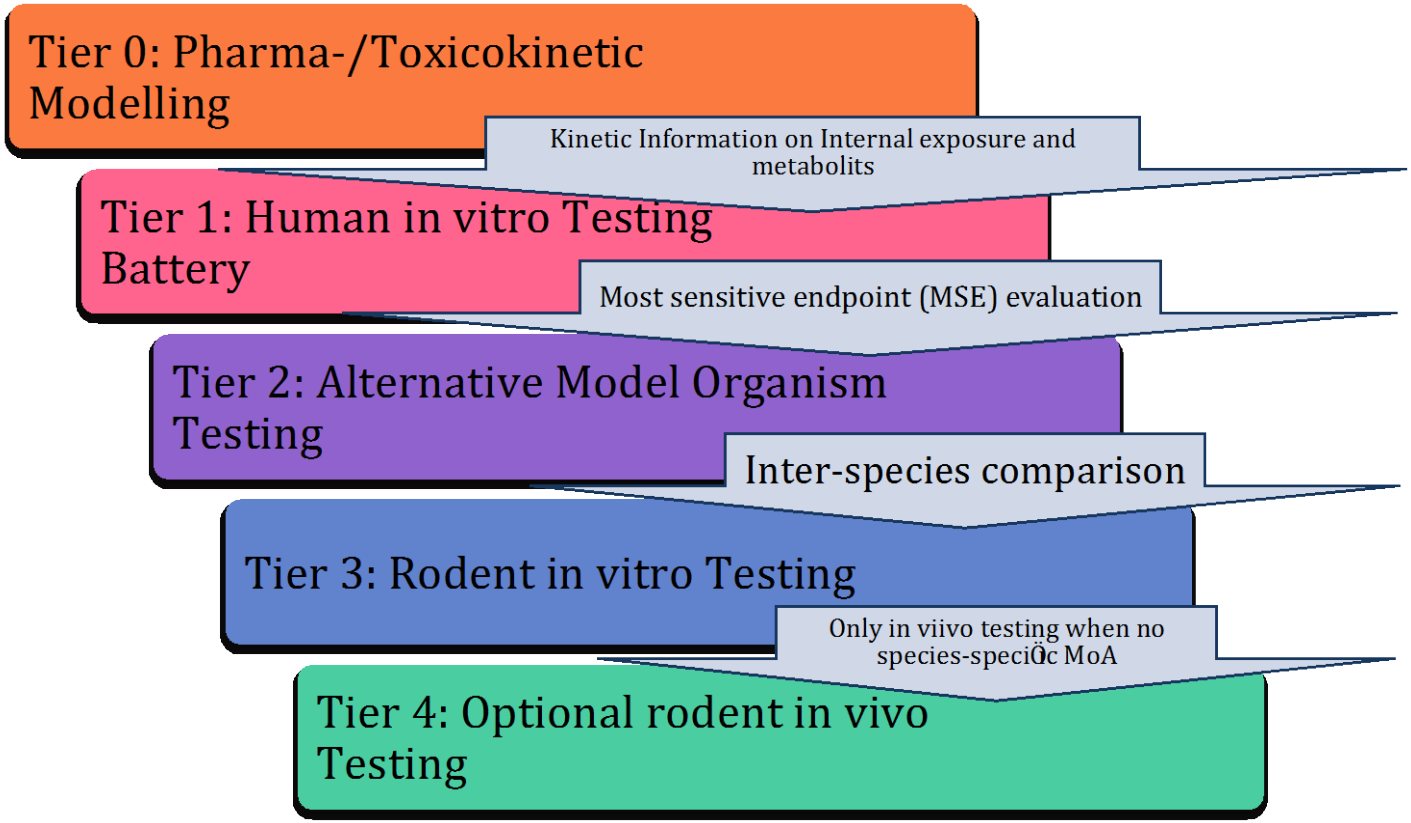
- In vitro only
- Suggest not all are the same
- Does not contain MEAs or zebrafish endpoints



Courtesy of W. Mundy

Progress to Date
Tiered Testing & Decision Frameworks

Proposed tiered DNT testing :

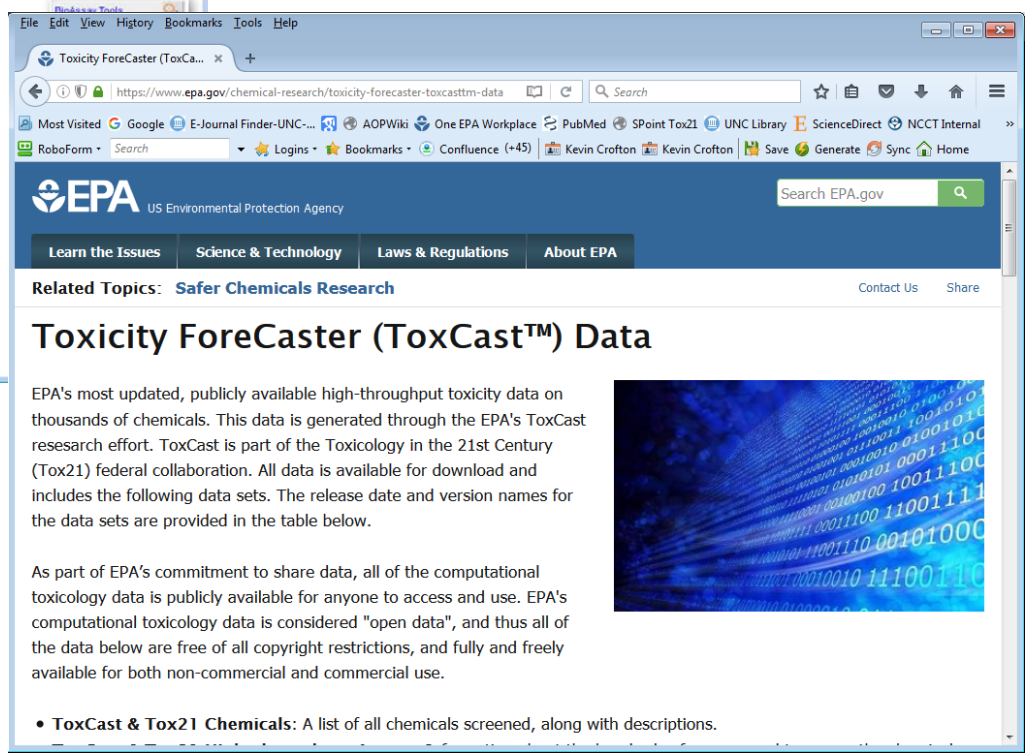


Fritsche, 2016

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*


Thanks for Listening