Oklahoma State University
Interdisciplinary Toxicology Program Symposium

Reducing the Use of Animals in Toxicity Testing: The In Vitro Developmental Neurotoxicity Testing Battery

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Disclosure Statement

This work has been funded by the US. Environmental Protection Agency. I have no conflicts to declare.

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Outline

I. The Need for Alternative Approaches for Developmental Neurotoxicity Hazard Assessment

II. The DNT In vitro battery (DNT-IVB)

III. Applying the DNT-IVB

IV. Future Directions

V. Questions

Requirements of EPA 870.6300 (OECD TG 426/443)

- 6 Pregnant females/dose (20 litters/dose recommended)
- 10 pups/litter (5 male/5 female)
- Minimum 3 doses + control
- Dosing period GD6-PND10
- Assessments on PND 4, 11, 21, 35, 45, 60

- Signs of Maternal Toxicity
- Developmental landmarks
- Brain/body weights (4, 11, 17, 21 PND)
- Motor activity (13, 17, 21, 60 PND)
- Auditory Startle (weaning, PND 60)
- Learning and memory (weaning, PND 60)
- Neuropathology (PND 11 and termination)
  - Major brain regions

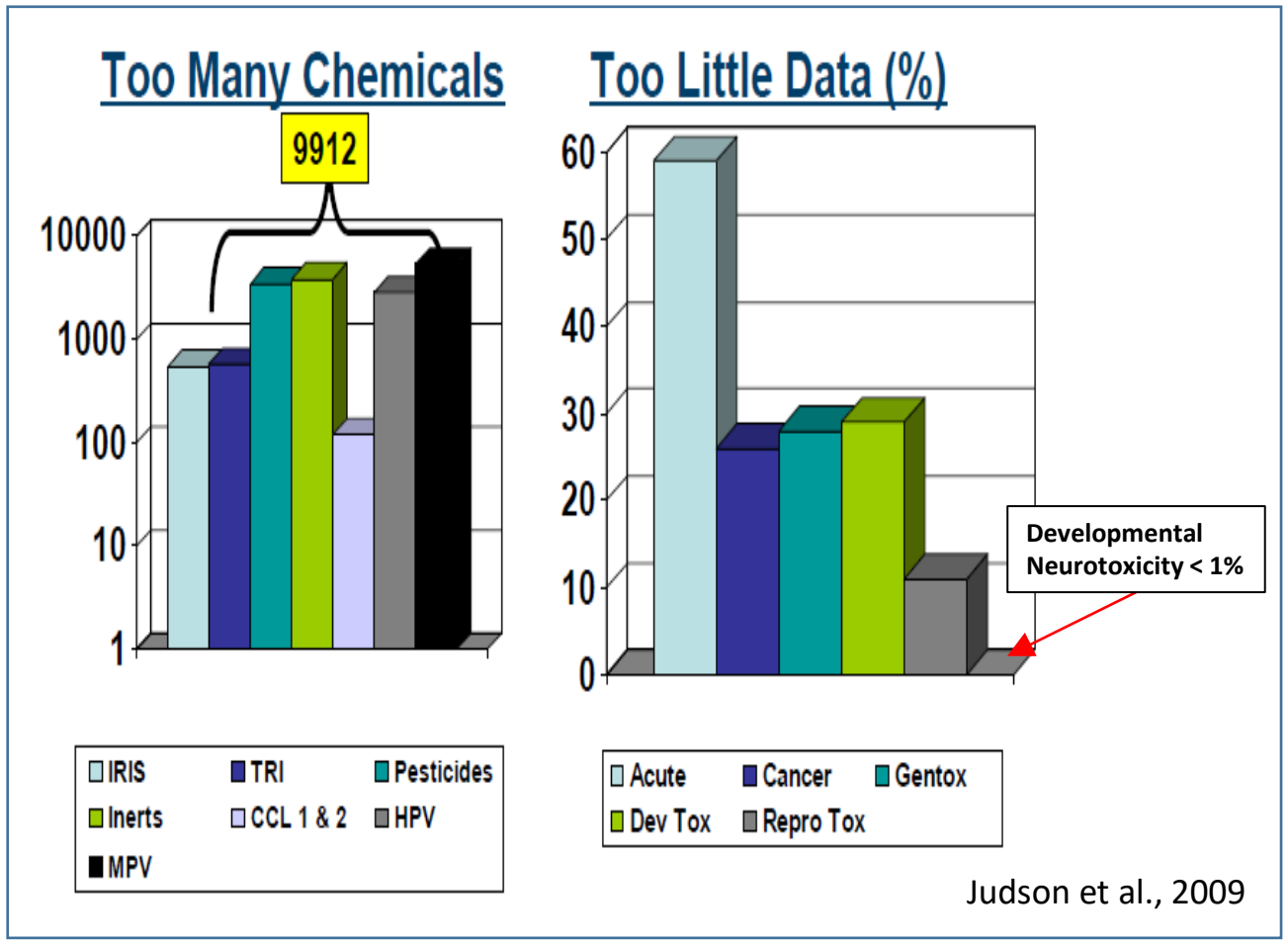
https://beta.regulations.gov/document/EPA-HQ-OPPT-2009-0156-0042
https://www.oecd-ilibrary.org/environment/test-no-426-developmental-neurotoxicity-study_9789264067394-en
https://www.oecd.org/chemicalsafety/test-no-443-extended-one-generation-reproductive-toxicity-study-9789264185371-en.htm

Issues with *in vivo* DNT studies

- "Triggered" test- Only requested if concern for neurotoxicity
- Expensive- ~$1,000,000/chemical
- Time-consuming- takes 1-2 years to complete
- Ethically questionable- Estimated ~1000 animals/test
- Value of Information
  - Quality of data varies considerably
  - Not often used for point of departure values for risk assessment*

*Raffaele et al. The use of **developmental neurotoxicity** data in pesticide risk assessments. Neurotoxicol Teratol. 2010 Sep-Oct;32(5):563-72.

Many Chemicals Lack Developmental Neurotoxicity (DNT) Data



Judson et al., 2009

Current testing too slow

- Not Required under FIFRA or TSCA
- Animal "Guideline" DNT; 1 chemical, $1M cost; 2 yr
- At current pace, ~150 chemicals in 20+ yrs
- Not often used (~25%) for point of departure values for risk assessment*

The absence of DNT hazard data on chemicals impedes consideration of this adverse outcome in environmental decision-making.

Reports of the potential involvement of environmental chemicals in increased rates of neurodevelopmental disease contributed to increasing public concern about DNT hazard of chemicals

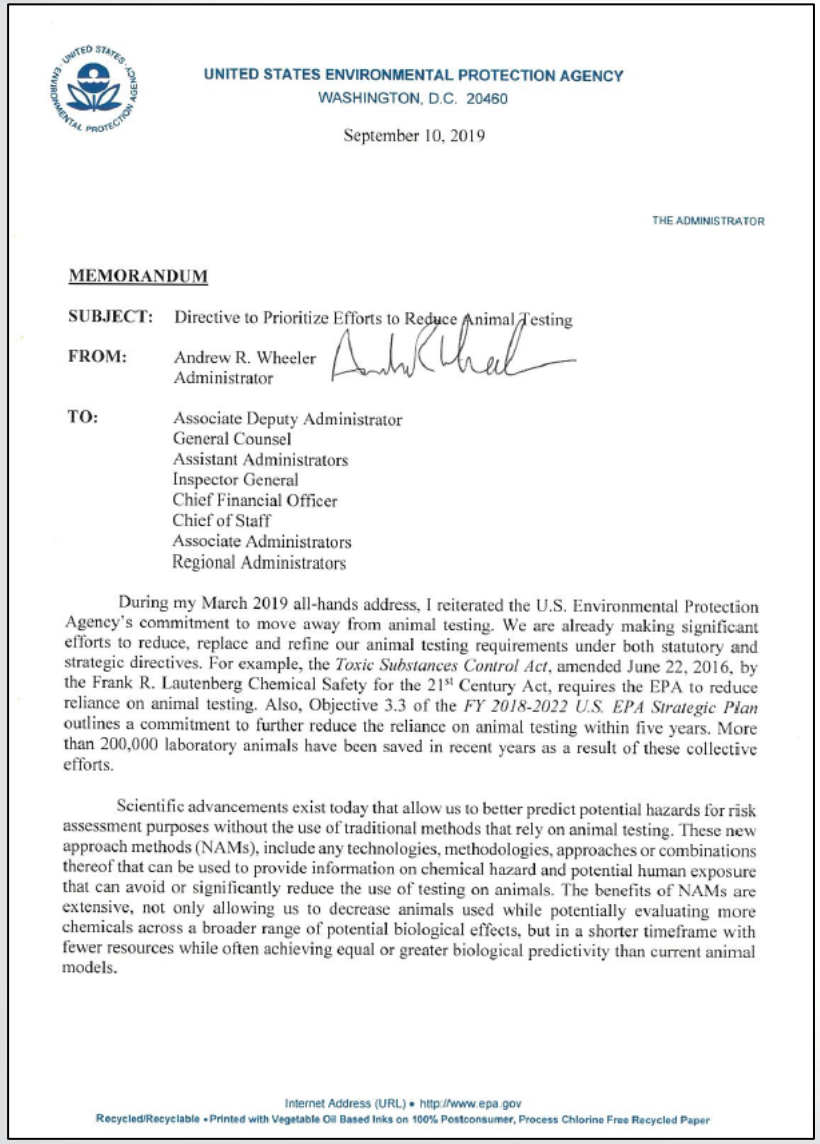
*Raffaele et al. The use of **developmental neurotoxicity** data in pesticide risk assessments. Neurotoxicol Teratol. 2010 Sep-Oct;32(5):563-72.

Solution: Faster, inexpensive and predictive methods are needed to detect and characterize compounds with developmental neurotoxicity hazard

- Develop high throughput, *in vitro* assays,
- Characterize chemicals for developmental neurotoxicity hazard
- Use human models whenever possible

- Data from these assays can provide information for decision-making

Drivers for NAMs



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

September 10, 2019

THE ADMINISTRATOR

MEMORANDUM

SUBJECT: Directive to Prioritize Efforts to Reduce Animal Testing

FROM: Andrew R. Wheeler
Administrator

TO: Associate Deputy Administrator
General Counsel
Assistant Administrators
Inspector General
Chief Financial Officer
Chief of Staff
Associate Administrators
Regional Administrators

During my March 2019 all-hands address, I reiterated the U.S. Environmental Protection Agency's commitment to move away from animal testing. We are already making significant efforts to reduce, replace and refine our animal testing requirements under both statutory and strategic directives. For example, the *Toxic Substances Control Act*, amended June 22, 2016, by the Frank R. Lautenberg Chemical Safety for the 21st Century Act, requires the EPA to reduce reliance on animal testing. Also, Objective 3.3 of the *FY 2018-2022 U.S. EPA Strategic Plan* outlines a commitment to further reduce the reliance on animal testing within five years. More than 200,000 laboratory animals have been saved in recent years as a result of these collective efforts.

Scientific advancements exist today that allow us to better predict potential hazards for risk assessment purposes without the use of traditional methods that rely on animal testing. These new approach methods (NAMs), include any technologies, methodologies, approaches or combinations thereof that can be used to provide information on chemical hazard and potential human exposure that can avoid or significantly reduce the use of testing on animals. The benefits of NAMs are extensive, not only allowing us to decrease animals used while potentially evaluating more chemicals across a broader range of potential biological effects, but in a shorter timeframe with fewer resources while often achieving equal or greater biological predictivity than current animal models.

Internet Address (URL) • http://www.epa.gov
Recycled/Recyclable •Printed with Vegetable Oil Based Inks on 100% Postconsumer, Process Chlorine Free Recycled Paper

Lautenberg Amendment to TSCA requires EPA to assess more chemicals

- Authorizes the use of alternative approaches

Under FIFRA, Office of Pesticides wants more and better information regarding DNT

USEPA Administrator Memo Prioritizing Efforts to Reduce Animal Testing, September 10, 2019

- EPA will reduce its requests for, and our funding of, mammal studies by 30 percent by 2025
- EPA will eliminate all mammal study requests and funding by 2035.
- Form a working group of agency experts in this field who will provide a work plan within six months.
- https://www.epa.gov/environmental-topics/administrator-memo-prioritizing-efforts-reduce-animal-testing-september-10-2019

In Europe, REACH legislation requires data on all compounds used in commerce

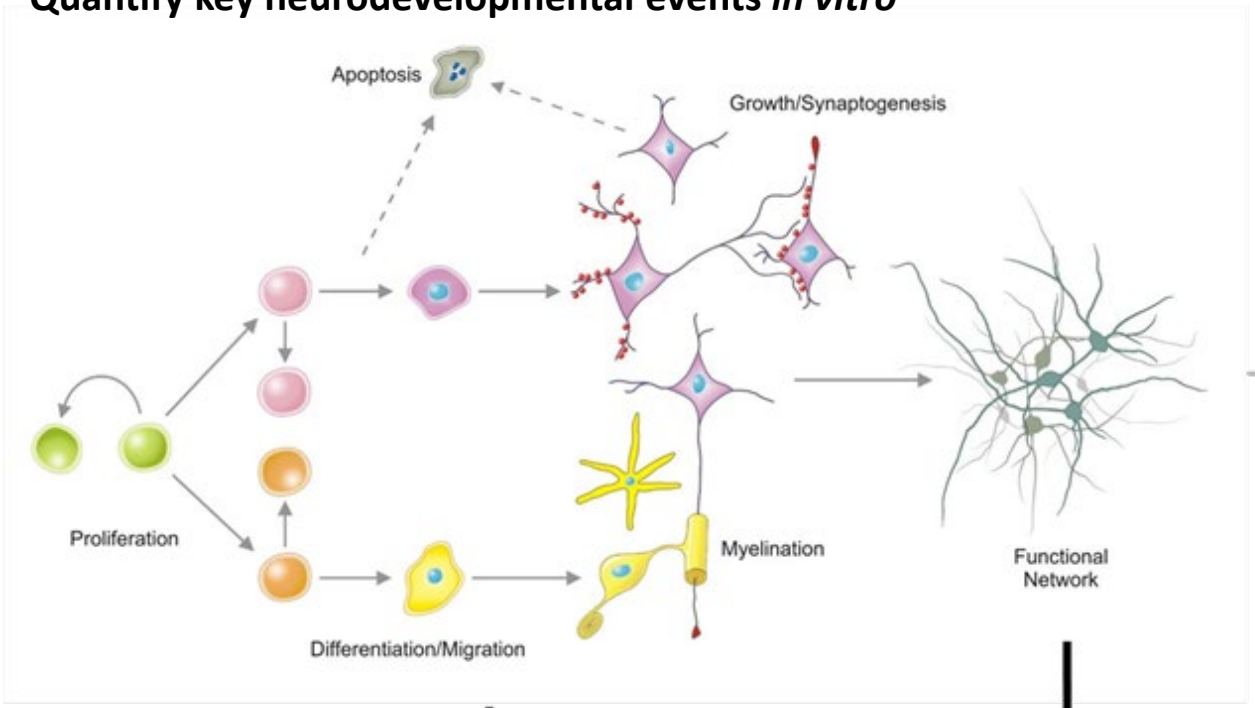
Challenges to Development of DNT Screens

- Central nervous system development is complex
  - Multiple potential targets
  - Time-dependent processes
  - Spatially-dependent processes
- Which target? Where? When?

Therefore, focus research on *key neurodevelopmental processes*

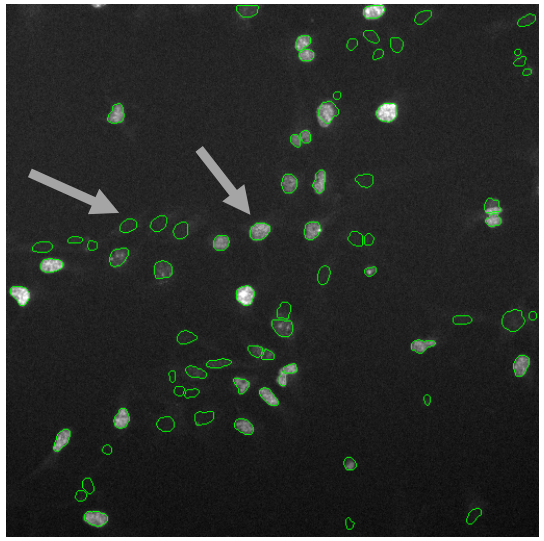
Phenotypic Screening for DNT Hazard

Quantify key neurodevelopmental events *in vitro*

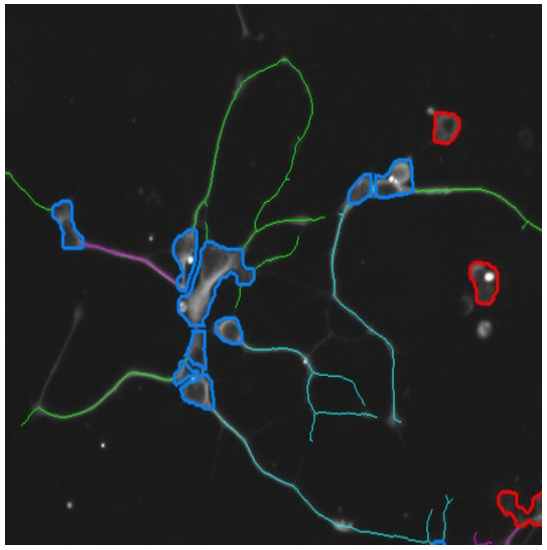


EPA DNT NAM Assays

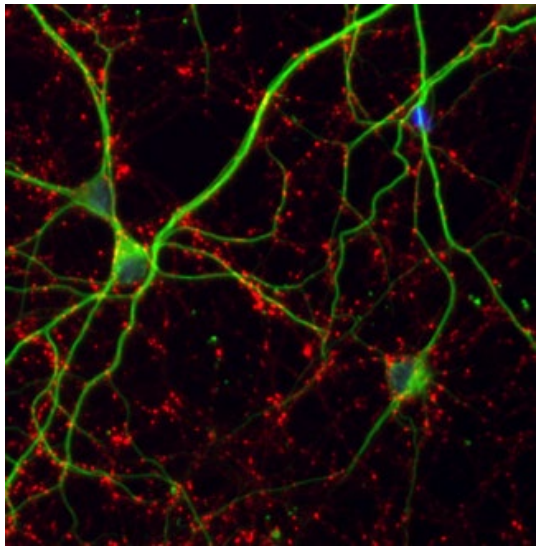
Proliferation



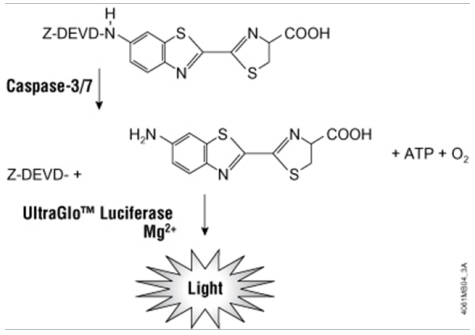
Neurite Outgrowth



Synaptogenesis

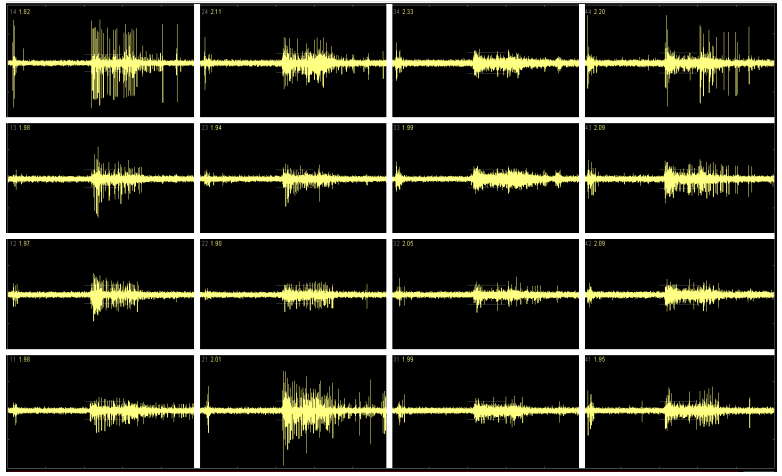


Apoptosis



luminescence

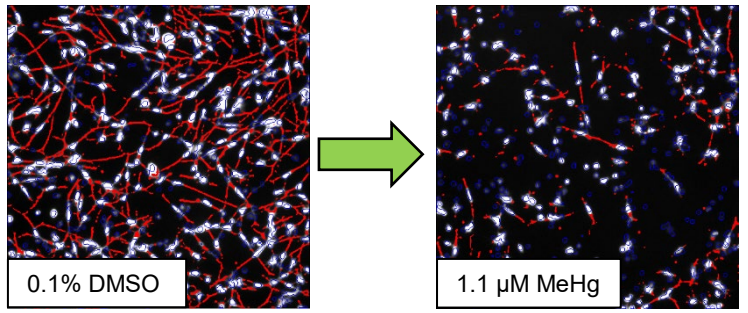
Network Function and Formation



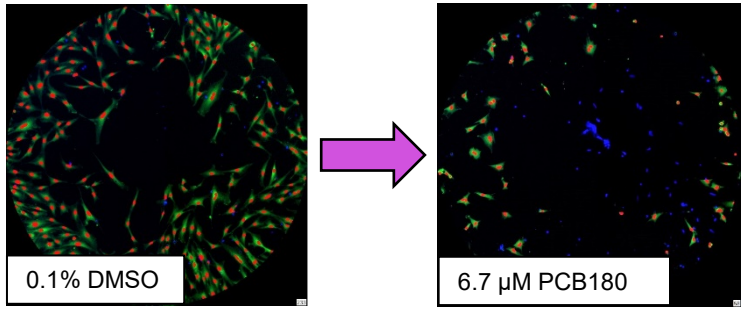
University of Konstanz NAM Assays

DNT compounds

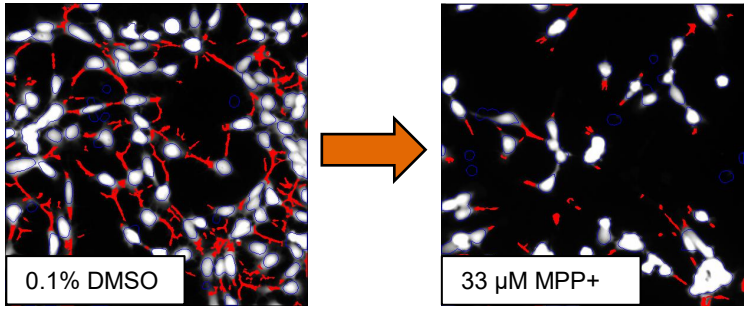
Effect on neurite outgrowth of PNS neurons

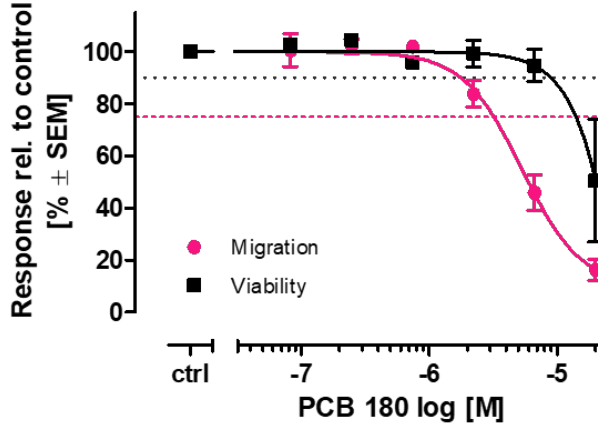


Effect on migration of neural crest cells



Effect on neurite outgrowth of CNS neurons

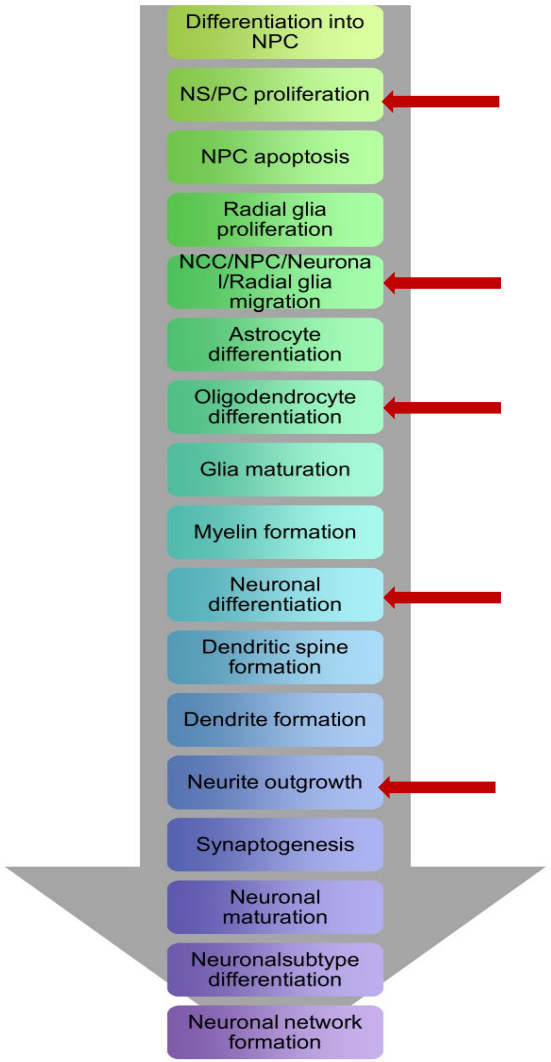


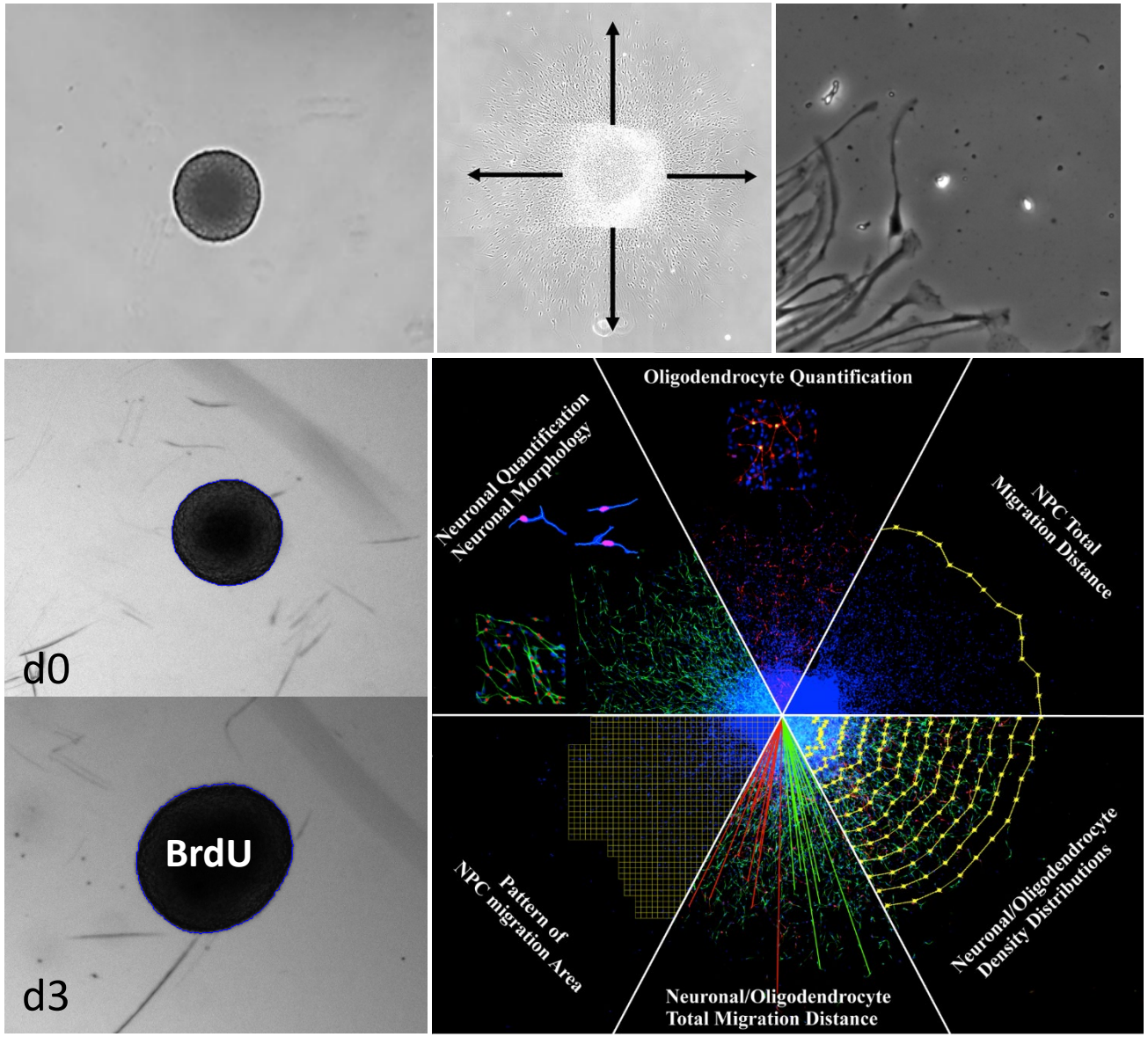


Prediction model & hit definition

Slide courtesy of M. Leist

The 'Neurosphere Assay' (Düsseldorf)





Baumann *et al.*, 2015 Methods in Pharmacology and Toxicology
Schmuck *et al.*, 2016 Archives of Toxicology
Masjosthusmann et al 2018 Toxicology and Applied Pharmacology

Slide courtesy of E. Fritsche

BrdU= bromdesoxyuridine

International Efforts on DNT NAMs

OXFORD SOT Society of Toxicology
www.toxsci.oxfordjournals.org

TOXICOLOGICAL SCIENCES, 167(1), 2019, 45–57

doi: 10.1093/toxsci/kfy211
Advance Access Publication Date: November 23, 2018
Forum

FORUM

International Regulatory and Scientific Effort for Improved Developmental Neurotoxicity Testing

Magdalini Sachana,*,[1] Anna Bal-Price,† Kevin M. Crofton,‡ Susanne H. Bennekou,§ Timothy J. Shafer,¶ Mamta Behl,‖ and Andrea Terron‖

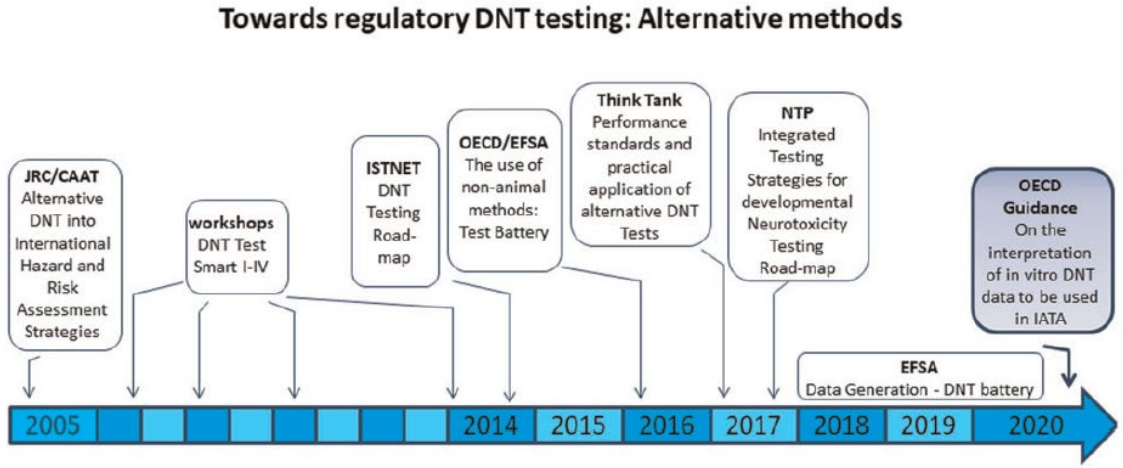
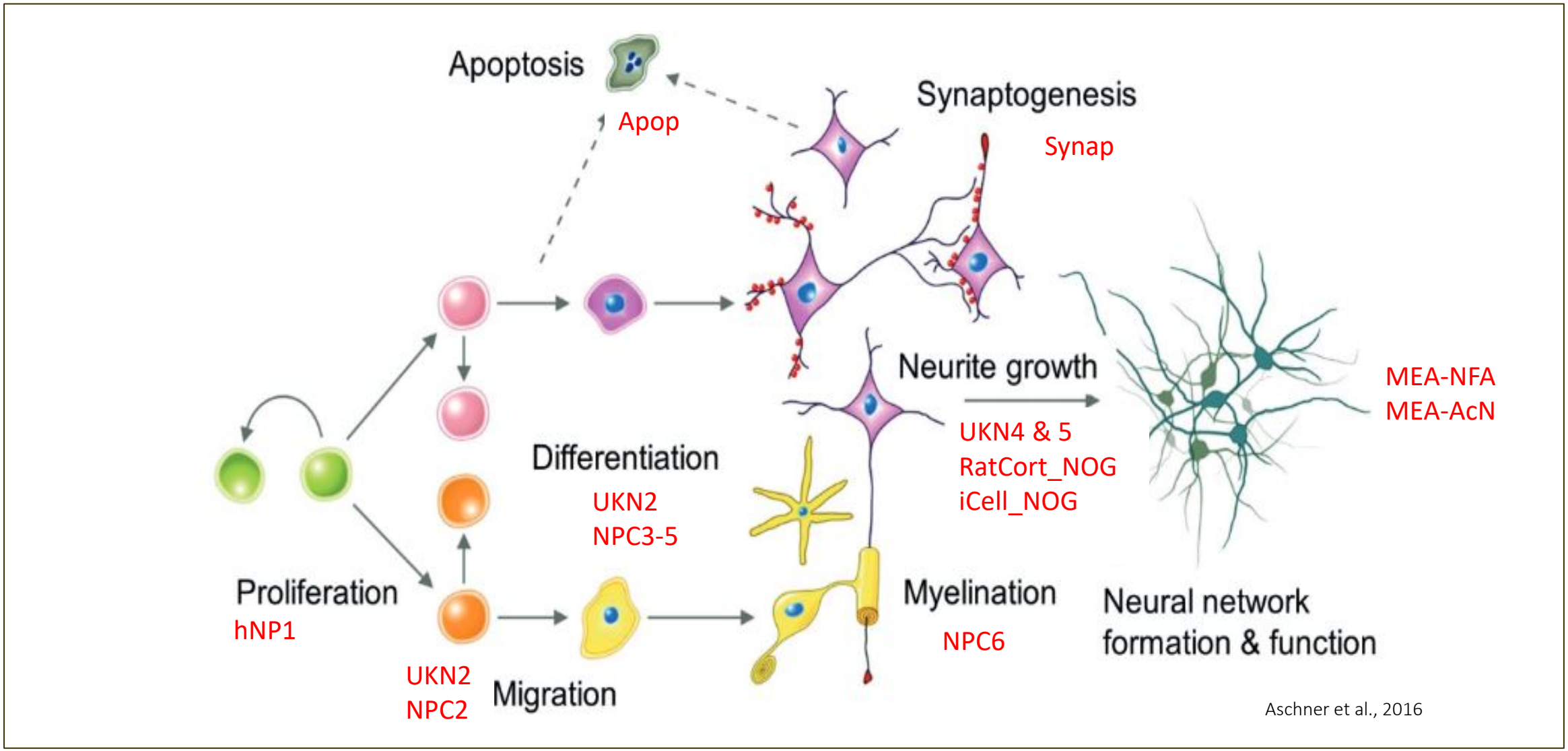


Figure 1. Timeline of efforts to develop and implement new alternative methods for developmental neurotoxicity.

Table 2. Proposed Assays for Evaluation As an *In Vitro* DNT Battery

Process	Assays	References
Proliferation	hNP1	Harrill *et al.* (2018)
	NPC1	Baumann *et al.* (2016) and Barenys *et al.* (2017)
	UKN1	Balmer *et al.* (2012)
Apoptosis	hNP1	Harrill *et al.* (2018)
Migration	NPC2	Baumann *et al.* (2016) and Barenys *et al.* (2017)
	UKN2	Nyffeler *et al.* (2017)
Neuron differentiation	NPC3	Baumann *et al.* (2016) and Barenys *et al.* (2017)
Oligodendrocyte differentiation & maturation	NPC5/6	Baumann *et al.* (2016) and Barenys *et al.* (2017)
Neurite outgrowth	iCell gluta hN2	Harrill *et al.* (2018)
	UKN 4 & 5	Krug *et al.* (2013)
	NPC4	Baumann *et al.* (2016) and Barenys *et al.* (2017)
Synaptogenesis	Rat primary synaptogenesis	Harrill *et al.* (2018)
Network formation	MEA-NFA	Brown *et al.* (2016) and Frank *et al.* (2018)

DNT NAMs Coverage of Neurodevelopmental Processes



Aschner et al., 2016

Encouraging Regulatory Use of NAMs

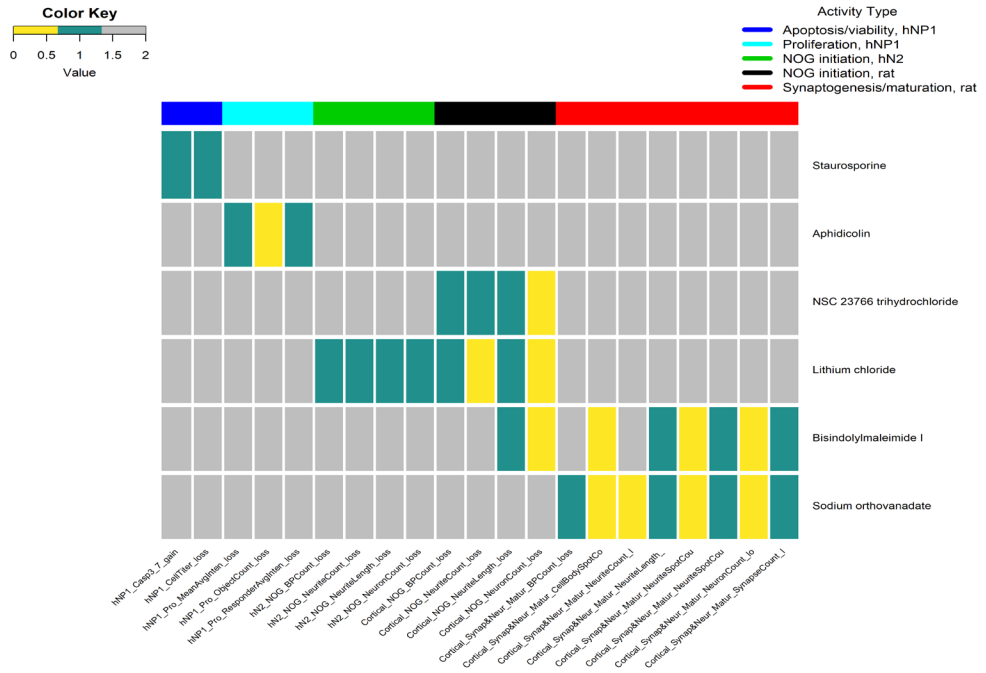
- Understanding of how the assays work and what they measure
- Evaluation of individual assays and the battery of assays
- Data from alternative assays
- Understanding of what can be done with the data
- Accessibility to the data

Regulatory decision-makers must have confidence in the assays and data in order to incorporate them into the decision-making process

Evaluating the Performance of DNT NAMs

Several different approaches can be taken to evaluate the performance of the DNT-NAMS

- Demonstrate that they recapitulate the *in vivo* neurobiology
- Evaluate Performance of Positive Controls
- Evaluate the Reproducibility when retesting compounds
- Evaluation of the Sensitivity/Specificity



True Positive Rate (sensitivity) = True positives/Known Positives

True Negative Rate (specificity) = True negatives/Known Negatives

Precision = True positives/(True Positives + False Positives)

Accuracy = (True Positives + True Negatives)/(Known Positives + Known Negatives)

The Challenge of Evaluating DNT NAMs Sensitivity and Specificity

NTP Report on Human Carcinogens (2016)

- 62 recognized, human carcinogens
- >170 “Anticipated” human Carcinogens
- >1000 compounds evaluated

By Contrast, for DNT:

- 12 recognized human developmental neurotoxicants (Grandjean and Landrigan, Lancet Neurol. 2014).
- ~150 compounds evaluated in Guideline DNT studies (rodents).

As a result, benchmarking the performance of *in vitro* DNT assays against *in vivo* data is confounded, but still needs to be done at some level

Goal: Assess the level of information in the literature that a chemical has *DNT hazard* or does not cause DNT

Evaluating 500 putative "DNT Reference" Compounds..

Neurotoxicology and Teratology 52 (2015) 25–35

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

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

Review article

Expanding the test set: Chemicals with potential to disrupt mammalian brain development

William R. Mundy [a,*], Stephanie Padilla [a], Joseph M. Breier [a,1], Kevin M. Crofton [b], Mary E. Gilbert [a], David W. Herr [a], Karl F. Jensen [a], Nicholas M. Radio [a,2], Kathleen C. Raffaele [c], Kelly Schumacher [d], Timothy J. Shafer [a], John Cowden [b]

Identified ~100 compounds that have evidence of causing DNT in mammals

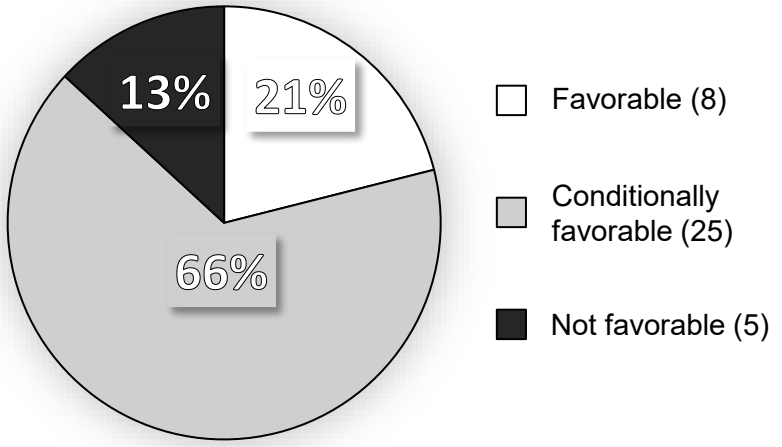
These are *scientific* summaries of evidence, not *regulatory* decisions. Summaries reflect *hazard*, not exposure or risk.

… and 38 putative "DNT Negative" Compounds

A.

Categorical definitions	
Favorable	Compounds in this category have convincing evidence for a lack of developmental neurotoxicity (DNT). If there are caveats, they are considered minor.
Conditionally favorable	Compounds in this category may be used as negative DNT reference compounds as there was no clear evidence that they cause DNT in vivo. However, caveats such as lack of (or conflicting) data, experimental design challenges, and/or chemistry/toxicokinetic uncertainties should be duly considered
Not favorable	These compounds have evidence that indicates they may have potential effects on the developing nervous system.

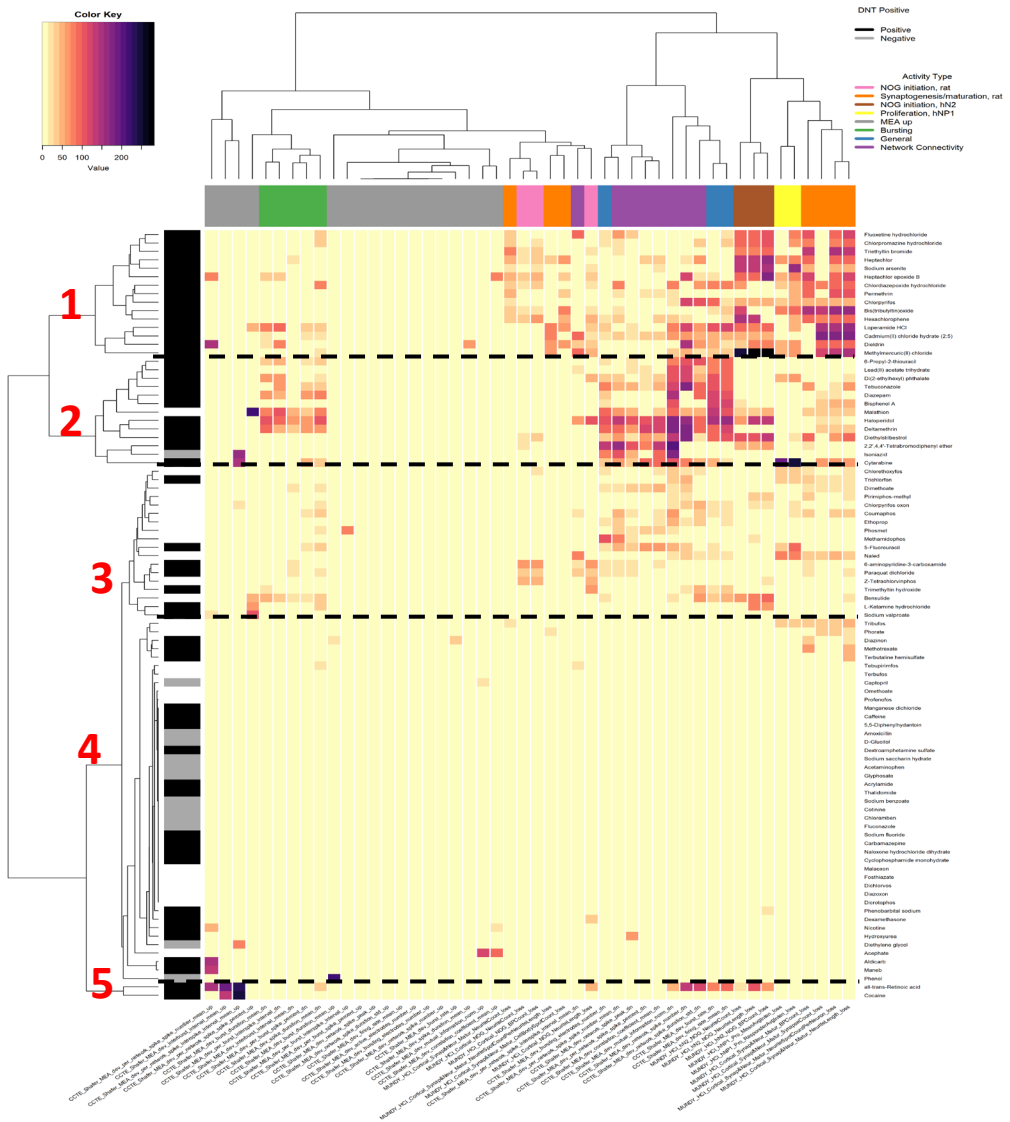
B.



https://doi.org/10.23645/epacomptox.14226326

Sensitivity/Specificity Analysis for EPA DNT NAMs

55 DNT Reference and 13 DNT Negative Compounds



	Strong selectivity	Moderate selectivity	DNT Reference: Negative	DNT Reference: Positive
1	Proliferation, synaptogenesis, NOG (hN2 cells)	NOG (rat cortical), firing rate, burst rate, and spike number	0	15
2	Decreased network formation activity	Synaptogenesis, and NOG (hN2 cells), decreased bursting activity	1 Isoniazid	11
3		Moderate to low activity across endpoints	0	7
4		Inactive/ equivocal	12	19
5	Increased mean inter-spike interval for network spikes		0	2

		Negatives	Positives
Results from DNT-NAM battery	**Selective activity** (Clusters 1,2,3,5)	False positive:1	True positive: 35
	Inactive/ equivocal (Cluster 4)	True Negative: 12	False negative: 19

Sensitivity= 65%, Specificity= 92%,

Carstens et al., in preparation

Encouraging Regulatory Use of DNT NAMs

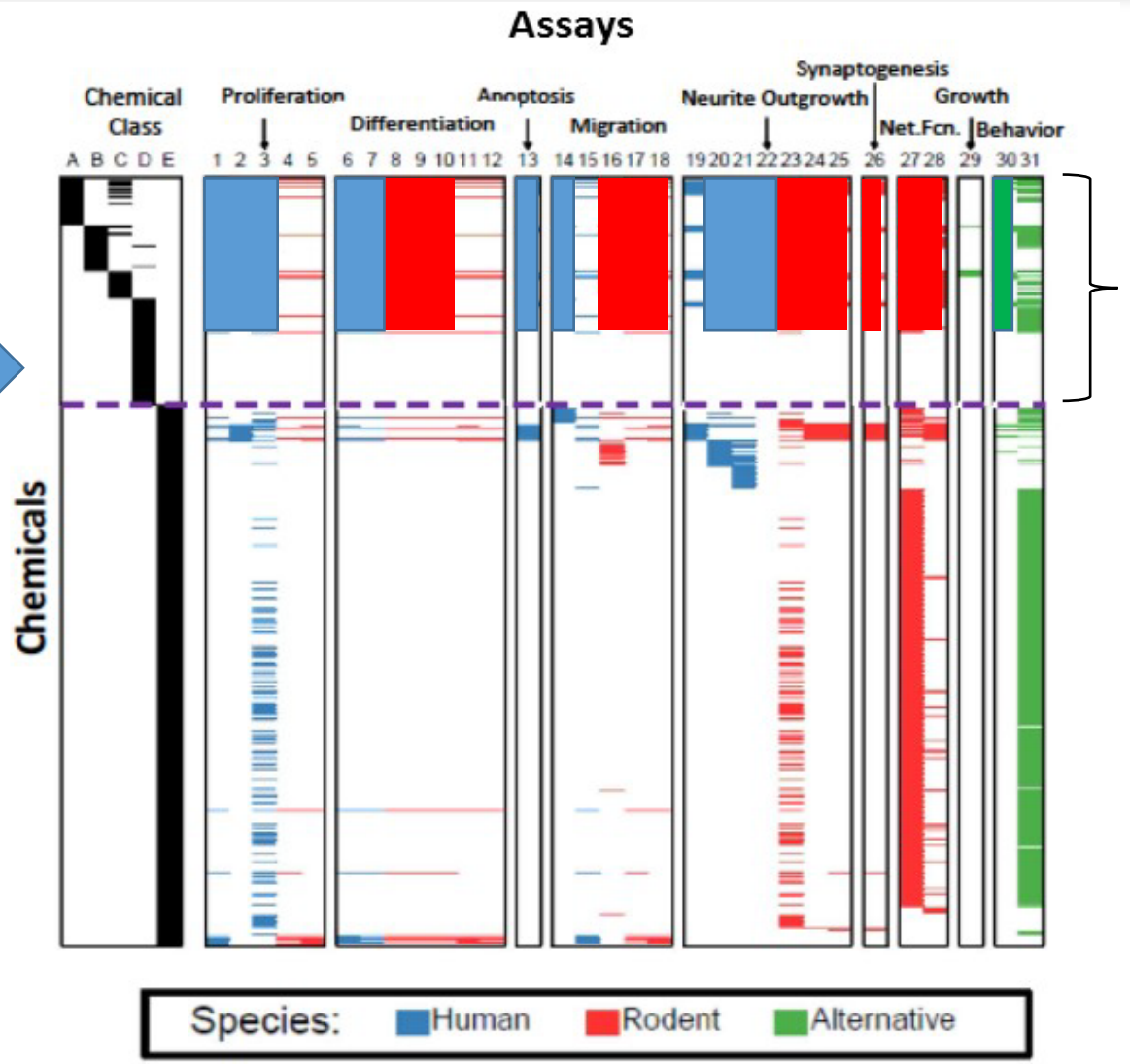
- Understanding of how the assays work and what they measure
- Evaluation of individual assays and the battery of assays
- Data from alternative assays
- Understanding of what can be done with the data
- Accessibility to the data

Regulatory decision-makers must have confidence in the assays and data in order to incorporate them into the decision-making process

The Need for More Data: Priority on compounds with *in vivo* DNT information

Assay-specific Compound Lists; Focused on *in vivo* DNT

Assay 1
Assay 2
Assay3…



Assay Evaluation

- Identified ~120 compounds:
  - Compounds for which DNT Guideline studies are available
  - Compounds of interest for Integrated Approaches to Testing and Assessment (IATAs)
  - Compounds where the Danish EPA has *in vivo* data
  - Negative compounds
  - Modulators of developmental pathways
- ToxCast has supplied most of these compounds

Bal-Price et al., 2018; Sachana et al., 2019

Status and Timelines

- Partners have received ToxCast compounds.
  - Testing is Completed at Konstanz and Duesseldorf
    - Report has been released to the public.
      - https://www.efsa.europa.eu/en/supporting/pub/en-1938
  - EPA testing is Completed
    - Data expected in Mid-2021
  - Zebrafish behavioral testing
    - Focus on ~30 IATA compounds
    - Data collection has started and will be completed later in 2021.

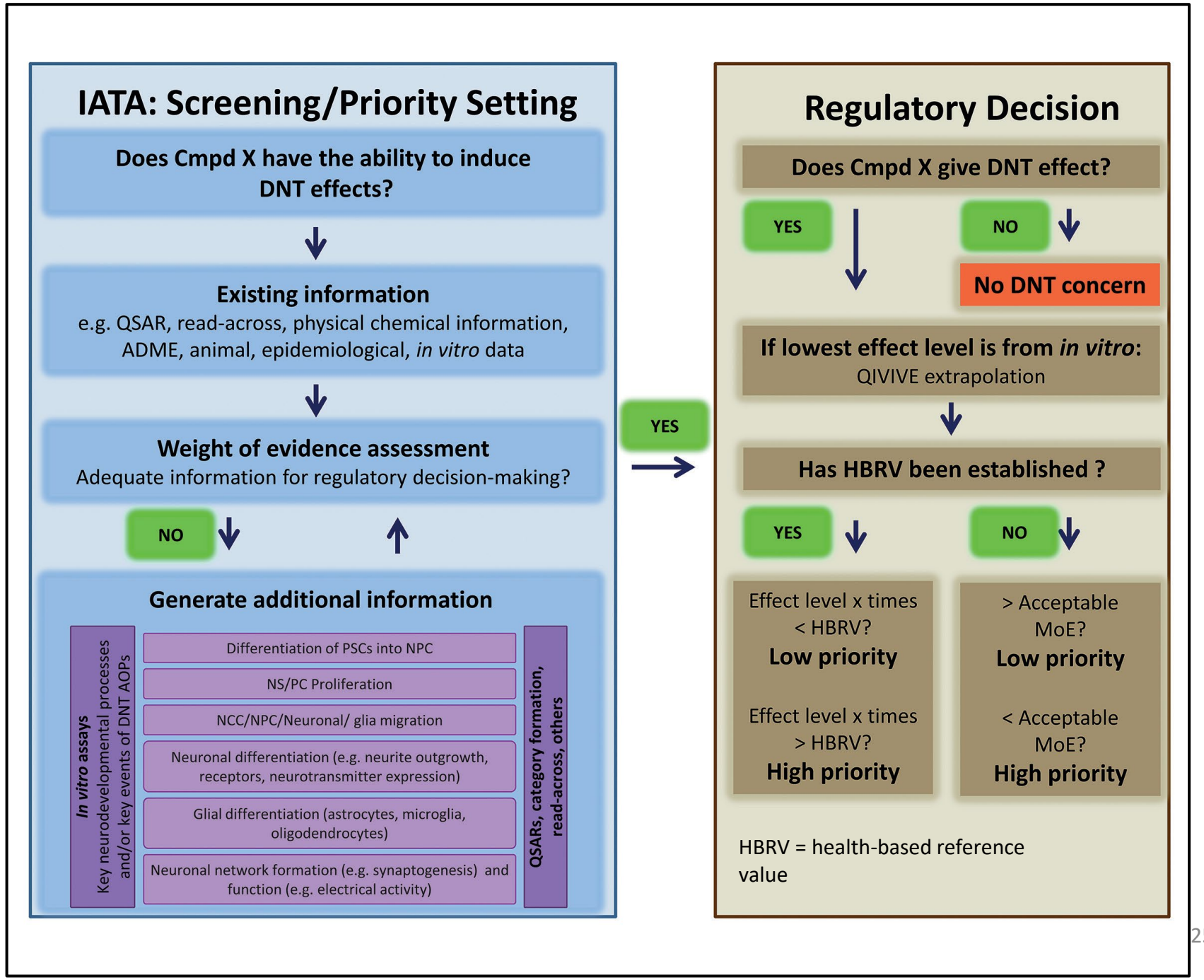
Encouraging Regulatory Use of DNT NAMs

- Understanding of how the assays work and what they measure
- Evaluation of individual assays and the battery of assays
- Data from alternative assays
  - Particularly for compounds that will be used for IATA case studies
- Understanding of what can be done with the data
- Accessibility to the data

Regulatory decision-makers must have confidence in the assays and data in order to incorporate them into the decision-making process

Development of a Guidance Document for the use of DNT alternative assays in Integrated Approaches for Testing and Assessment (IATAs)

- Introduction and Rationale
- Issues with the Current Guideline testing approaches
- Guidance for incorporation of *in vitro* assays into IATAs
- **Case Studies**



Use of DNT NAMs at EPA

I. **Screening Level information**
   - APCRA, TSCA, PFAS

II. **Understanding species differences**
   - Data from DNT NAMs provided to OPP to help understand rodent-human differences in response to chemicals since the battery has both rodent and human assays

III. **Structure-activity relationships**
   - OPP requested data from selected assays on a set of structurally similar compounds
     - A DNT Guideline study existed for one compound (“compound X”)
     - Assays were selected based on the of activity of compound X in Guideline Study.
     - Structurally similar compounds were tested in vitro
     - OPP will use the data from the in vitro screens in WOE approach to deciding whether to request DNT guideline studies on the other compounds (Decisions are in progress).

IV. **Weight of Evidence approaches**
   - Organophosphates

Organophosphates and DNT

Organophosphate insecticides are currently regulated based on inhibition of acetylcholinesterase (AChE).

Primary Questions:

1) Does the DNT battery indicate that regulation based on AChE inhibition may not be health protective?

2) Can data from the DNT battery contribute to a WOE approach for OPs?

Organophosphates and DNT

Study Design:

Test 27 Organophosphate insecticides in the EPA DNT assays

8 Parent/oxon pairs

Concentration-response up to 100 µM

Pipeline results through TCPL to generate AC_{50} values

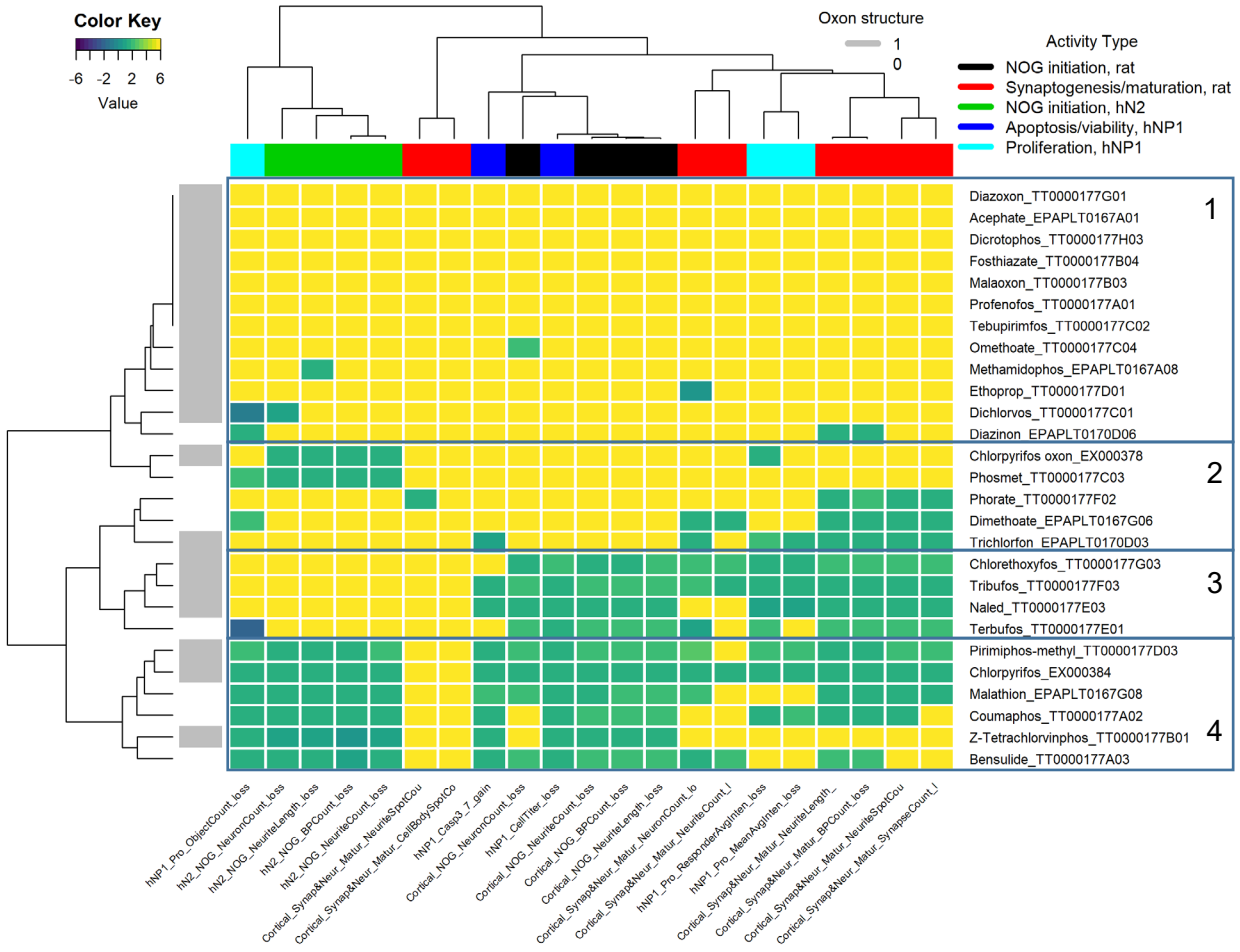
Use HTTK to convert AC_{50} values to AED_{50} values

Compare to BMD/BMDL10 values based on AChE inhibition

Assays:

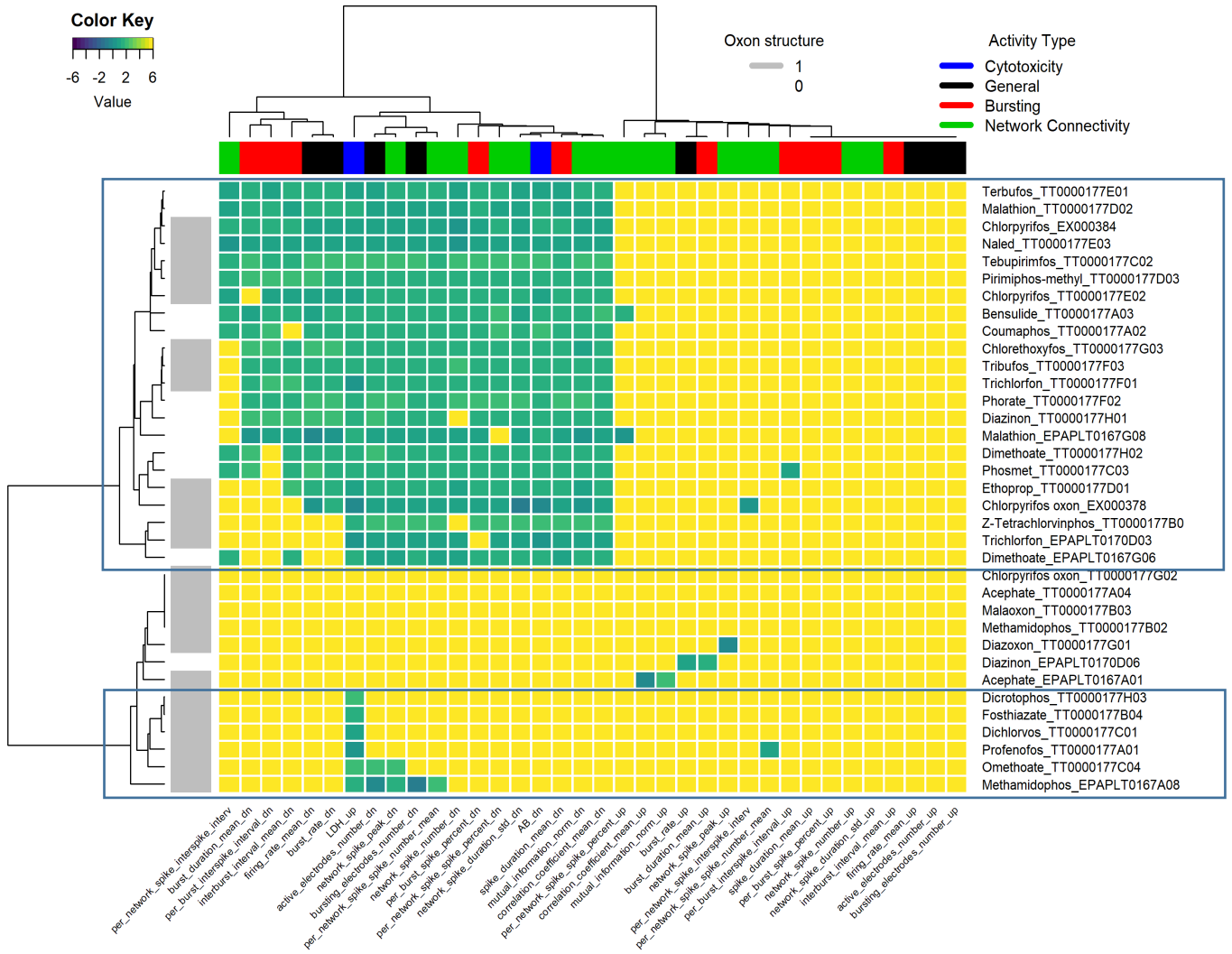
Proliferation	-	human neuroprogenitors (hNP1)
Apoptosis	-	human neuroprogenitors (hNP1)
Neurite initiation	-	human neurons (hN2)
Neurite initiation	-	rat primary neural culture
Neurite maturation	-	rat primary neural culture
Synaptogenesis	-	rat primary neural culture
Network formation (MEA)	-	rat primary neural culture
Behavior/Anatomy	-	zebrafish (data analysis pending)

OPs demonstrate differential responses in the HCI assays.



- Cluster 1: negative or with effects in 1-3 endpoints.
- Cluster 2: effects on 5 or more assay endpoints
- Cluster 3: OP samples with effects on all HCI assay activity types except for NOG initiation in hN2 cells and synaptogenesis in cortical cells
- Cluster 4: widespread effects across activity types

Most OPs decreased MEA NFA activity



- Top active cluster of OPs contains oxon and non-oxon structures.
- These OPs, like the assay performance controls and many other compounds, appear to generally decrease all activity types and most assay endpoints.
- Bottom cluster with minimal actives appears somewhat driven by cytotoxicity in the LDH assay.
- Negative- 0 assay endpoints altered
- Equivocal- 1-3 assay endpoints altered
- Positive- >3 assay endpoints altered

Agreement between the HCI and MEA_NFA assays

DTXSID	Chemical	MEA NFA			HCI			
		Neg	Equiv	Pos	1	2	3	4
DTXSID8023846	Acephate	X	X		X			
DTXSID9032329	Bensulide			X				X
DTXSID2032344	Chlorethoxyfos			X			X	
DTXSID4020458	Chlorpyrifos			X,X				X
DTXSID1038666	Chlorpyrifos oxon	X		X		X		
DTXSID2020347	Coumaphos			X				X
DTXSID9020407	Diazinon		X	X		X		
DTXSID5037523	Diazoxon		X		X			
DTXSID5020449	Dichlorvos		X		X			
DTXSID9023914	Dicrotophos		X		X			
DTXSID7020479	Dimethoate			X		X		
DTXSID4032611	Ethoprop			X	X			
DTXSID0034930	Fosthiazate		X		X			
DTXSID9020790	Malaoxon	X			X			
DTXSID4020791	Malathion			X				X
DTXSID6024177	Methamidophos	X	X			X		
DTXSID1024209	Naled			X			X	
DTXSID4037580	Omethoate		X		X			

DTXSID	Chemical	Neg	Equiv	Pos	1	2	3	4
DTXSID4032459	Phorate			X		X		
DTXSID5024261	Phosmet			X		X		
DTXSID0024266	Pirimiphos-methyl			X				X
DTXSID3032464	Profenofos		X		X			
DTXSID1032482	Tebupirimfos			X	X			
DTXSID2022254	Terbufos			X			X	
DTXSID1024174	Tribufos			X			X	
DTXSID0021389	Trichlorfon			X		X		
DTXSID1032648	Z-Tetrachlorvinphos			X				X

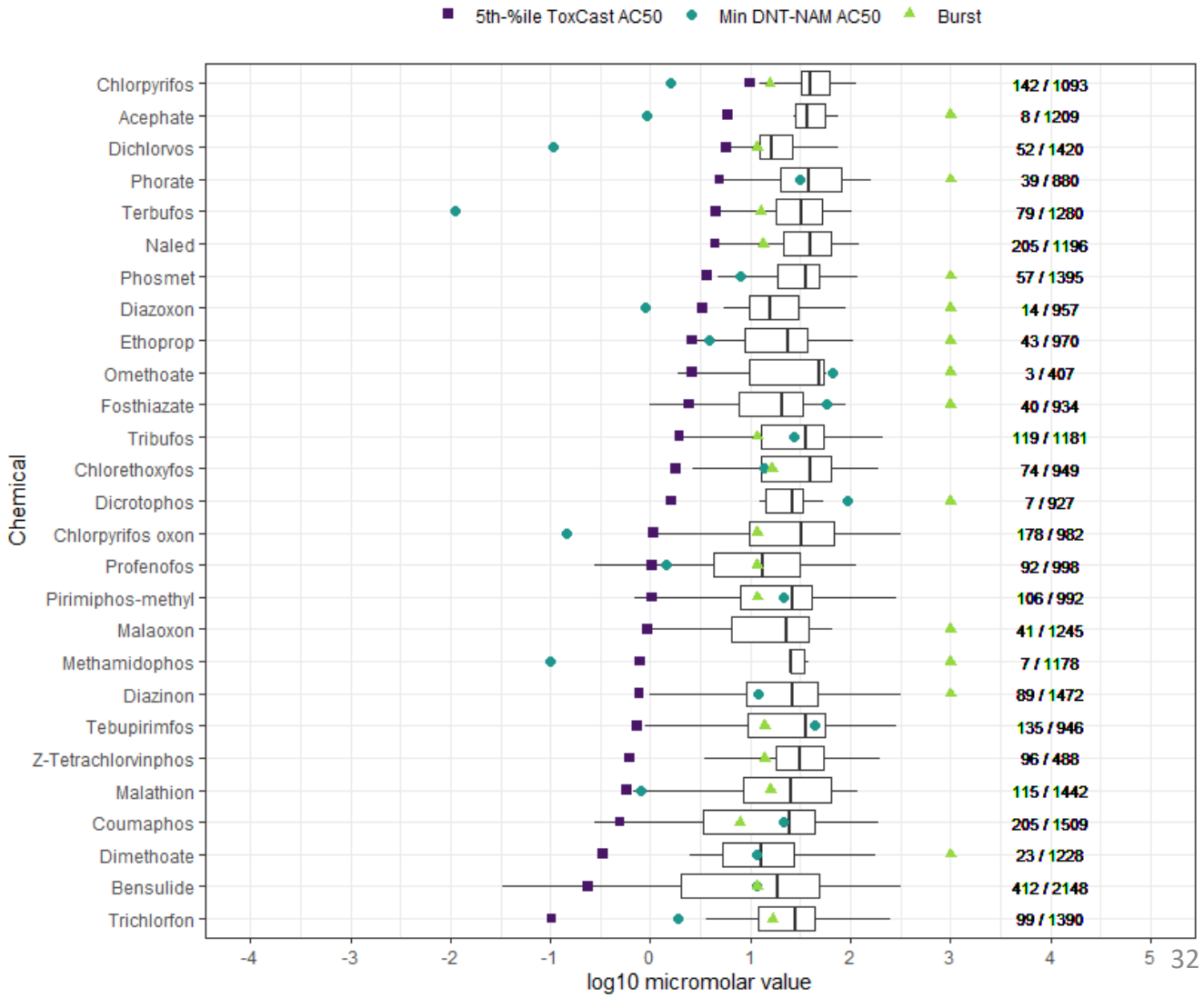
- *Equiv or Pos in MEA NFA and negative in HCI*: Acephate, diazoxon, dichlorvos, dicrotophos, fosthiazate, malaoxon, omethoate, profenofos
- *Positive in MEA NFA and negative in HCI*: Ethoprop
- *Positive in HCI and negative in MEA NFA:* OP chemical (methamidophos) was neg/equiv in the MEA NFA
- **If activity is observed in the HCI assays, it is likely that the OP chemical will also be active in the MEA NFA.**

For some OPs, DNT-NAM AC_{50} < bioactivity estimate from the rest of ToxCast.

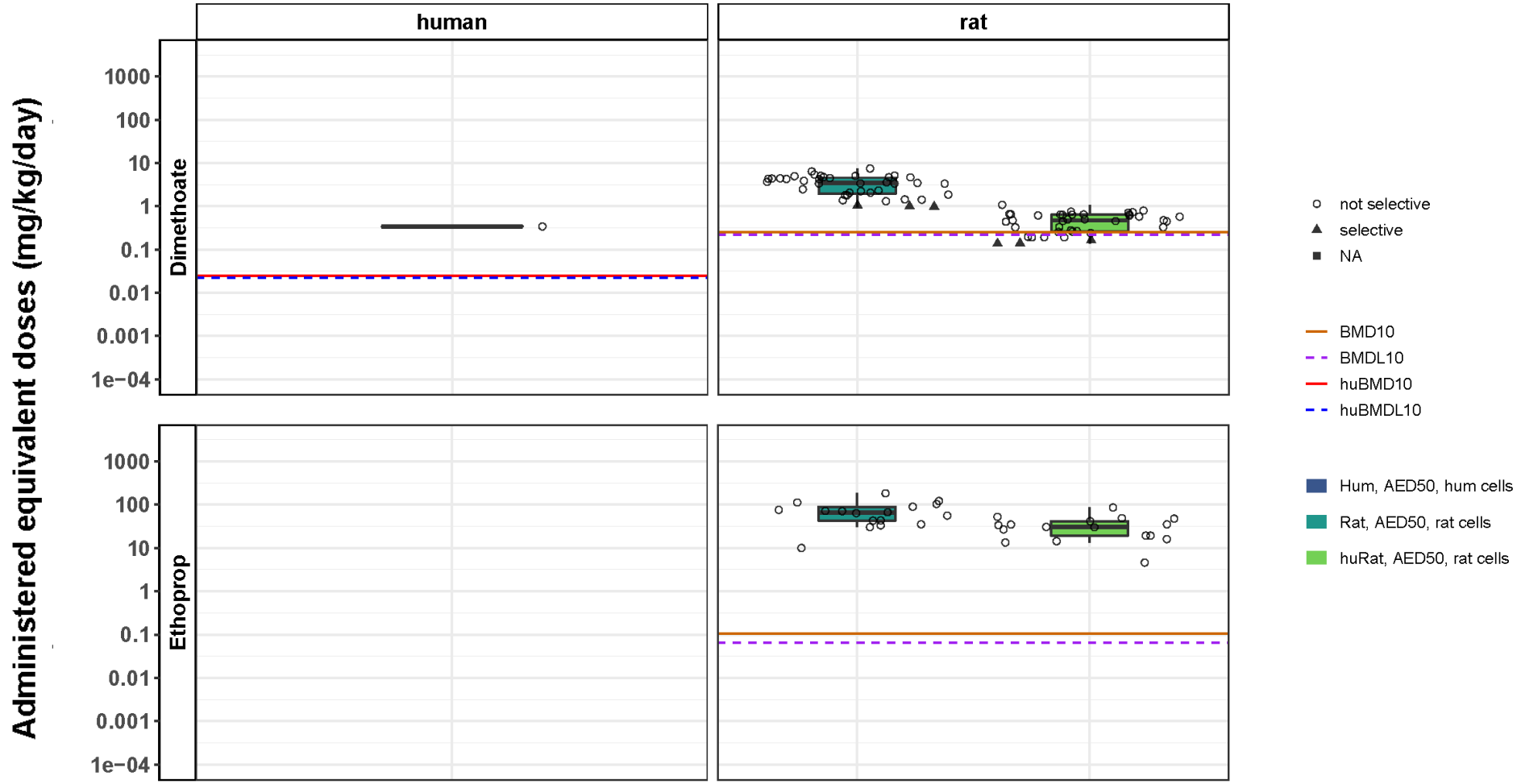
DNT-NAM battery may provide a more potent estimate of bioactivity for substances with minimum DNT-NAM AC50 < 5th percentile of filtered ToxCast AC50 values:

- Chlorpyrifos and chlorpyrifos oxon
- Acephate
- Dichlorvos
- Terbufos
- Diazoxon
- Methamidophos

Suggests that the DNT-NAM battery, in covering some new biology not previously in ToxCast, may yield bioactivity threshold concentrations lower than what is already available for some neuroactive substances in ToxCast.



AED50 to BMD/BMDL10 comparisons



Summary of the AED50 to BMD/BMDL comparison

	Chemicals with AED50 values >>> BMD/BMDL comparator	Chemicals with lowest AED50 within 1 log10 order of magnitude of BMD/BMDL comparator	Chemicals with lowest AED50 approaching BMD/BMDL comparator	Missing in vitro data for comparison
Rat/HuRat	Coumaphos, diazoxon, dicrotophos, ethoprop, fosthiazate, omethoate	acephate, bensulide, chlorpyrifos, chlorpyrifos oxon, diazinon, dimethoate, malathion, methamidophos, and phorate	dimethoate and methamidophos (lower quartile of huRat AED_{50} values dichlorvos (huRat AED_{50}; only one positive rat assay endpoint) overlaps with the BMDL10 value, and it was not based on selective bioactivity in the DNT-NAM battery. malathion (huRat AED_{50} (selective) for also approach the BMD/BMDL10 values.	Malaoxon (negative in all assays)
Human	bensulide, chlorpyrifos, chlorpyrifos oxon, coumaphos, diazinon, dimethoate, malathion, methamidophos, phosmet, pirimiphos-methyl, tribufos, and trichlorfon		dichlorvos, only two AED_{50} values are available for comparison, and these values are centered around the BMD10/10 and BMDL10/10 values. terbufos, only 3 human AED_{50} values are available for comparison, and the lowest one of these values approaches the BMD10/10 value.	**Negative in all assays with human cells:** Acephate, diazoxon, dicrotophos, ethoprop, fosthiazate, omethoate, phorate, profenofos, and tebupirimfos Malaoxon was negative in all assays.

AEDs from DNT NAMS can be more sensitive than LOAELs

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Evaluation of Chemical Effects on Network Formation in Cortical Neurons Grown on Microelectrode Arrays

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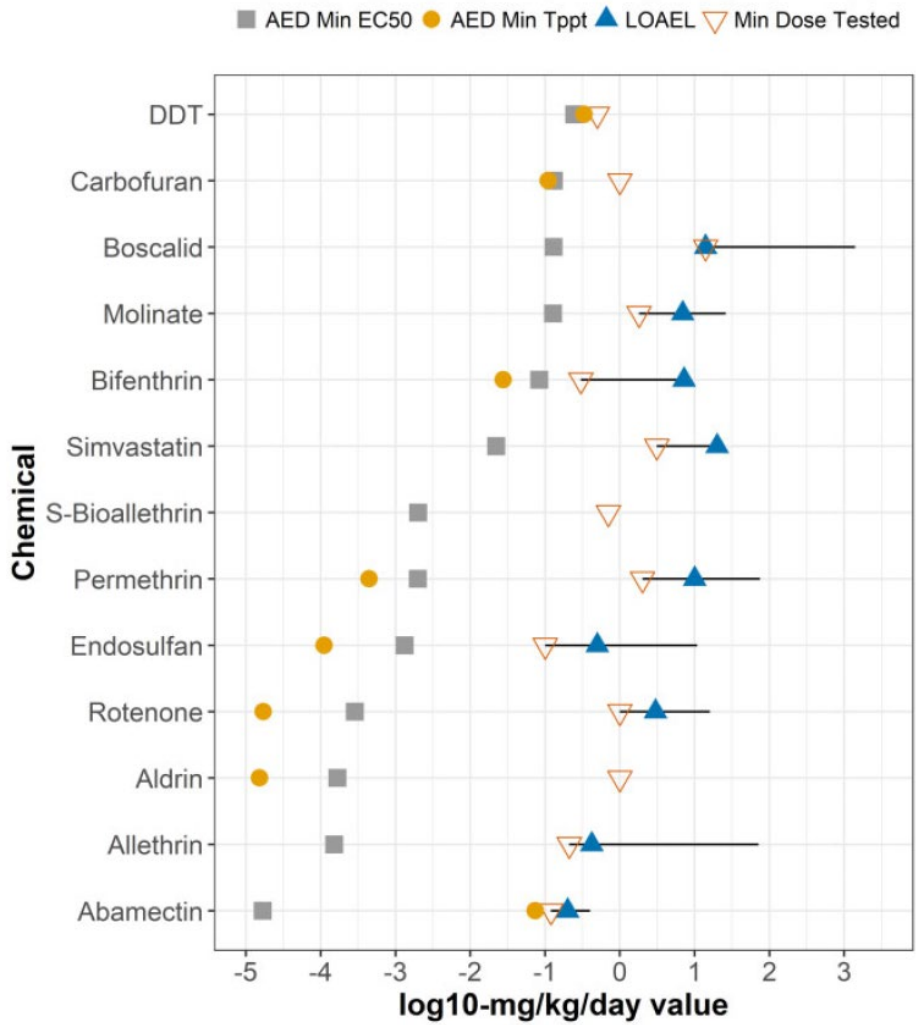

Even though AEDs were not more sensitive than BMDLs for OPs, DNT NAMs can still be sensitive indicators of potential disruption of nervous system development

In vitro assays to identify DNT Hazard:
Promises and challenges

Promises:

- Data on DNT hazard for many more chemicals
- Characterization of DNT hazard on biologically-relevant processes
- Data from human models
- Substantially lower cost and faster results than *in vivo* studies

Challenges/Future Directions:

- Further evaluation of the battery
- Development of additional case-studies using *in vitro* DNT assays
- Development of additional AOPs related to DNT that will increase confidence in using these assays
- Development of assays that cover areas of neurodevelopmental processes not well covered in the current battery

Thank you!
Questions?

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