Integration of New Approach Methodology (NAM) Data for Evaluating Potential Developmental Neurotoxicity

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Pesticide Registration

- Pesticides regulated in U.S. under Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and Federal Food, Drug and Cosmetics Act (FFDCA)
  - Both amended in 1996 by the Food Quality Protection Act (FQPA)
- 40 CFR Part 158 outlines data requirements for pesticides depending on expected use pattern
  - Food vs. non-food use
  - Routes and duration of exposure
- Toxicological studies provide information on wide range of adverse health outcomes, routes of exposure, duration, species, and lifestages

DNT Guideline

- Developmental neurotoxicity (DNT) refers to any adverse effect of exposure to a toxic substance on the normal development of nervous system structures and/or functions
- Test guidelines: OCSPP 870.6300 and OECD TG 426
- Conditionally required using weight of evidence (WOE) approach if:
  - The pesticide causes treatment-related neurological effects in adult animal studies
  - The pesticide causes treatment-related neurological effects in developing animals, following pre- and/or postnatal exposure
  - The pesticide elicits a causative association between exposures and adverse neurological effects in human epidemiological studies
  - The pesticide evokes a mechanism that is associated with adverse effects on the development of the nervous system

DNT Guideline

- Purpose: Designed to develop data on the potential functional and morphological hazards to the nervous system which may arise in the offspring from exposure of the mother during pregnancy and lactation
- Test substance administered to pregnant animals during gestation and early lactation
- Offspring randomly selected from within litters for neurotoxicity evaluation
  - Auditory startle, motor activity, functional observational battery (FOB), learning and memory, clinical observations, and neuropathological examinations

DNT Guideline

- 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
- Consider risk (hazard & exposure) when evaluating need for DNT study
- Registrants should submit any alternative proposed testing protocols and supporting scientific rationale to EPA prior to study initiation

DNT Guideline Challenges & Limitations

- Reliable detection, measurement, and interpretation of treatment-related DNT effects depends on appropriate study design and conduct
- Infers DNT effects on the basis of apical endpoints with little or no information on the underlying biological processes
- Interpretation hampered by a number of limitations including high variability, low precision, and being resource intensive
- Difficult to interpret isolated findings where a change in one endpoint is not substantiated by other endpoints
- Challenges correlating behavioral and/or neuropathological effects in the animal model to complex neurological deficits in the human population

Status of DNT Studies at OPP

- Approximately 100 DNT studies reviewed by OPP
- About 20 DNTs used to set points of departure (POD) for risk assessment
  - All based on offspring effects without corresponding maternal effects
    - Brain morphology
    - Behavioral changes
    - Pup mortality
    - Pup weight
    - Developmental delays
- 32 DNT studies available for known neurotoxicant chemical classes (OPs, *N*-methyl carbamates (NMCs), and pyrethroids)
  - None provided the most sensitive endpoint for human health risk assessment

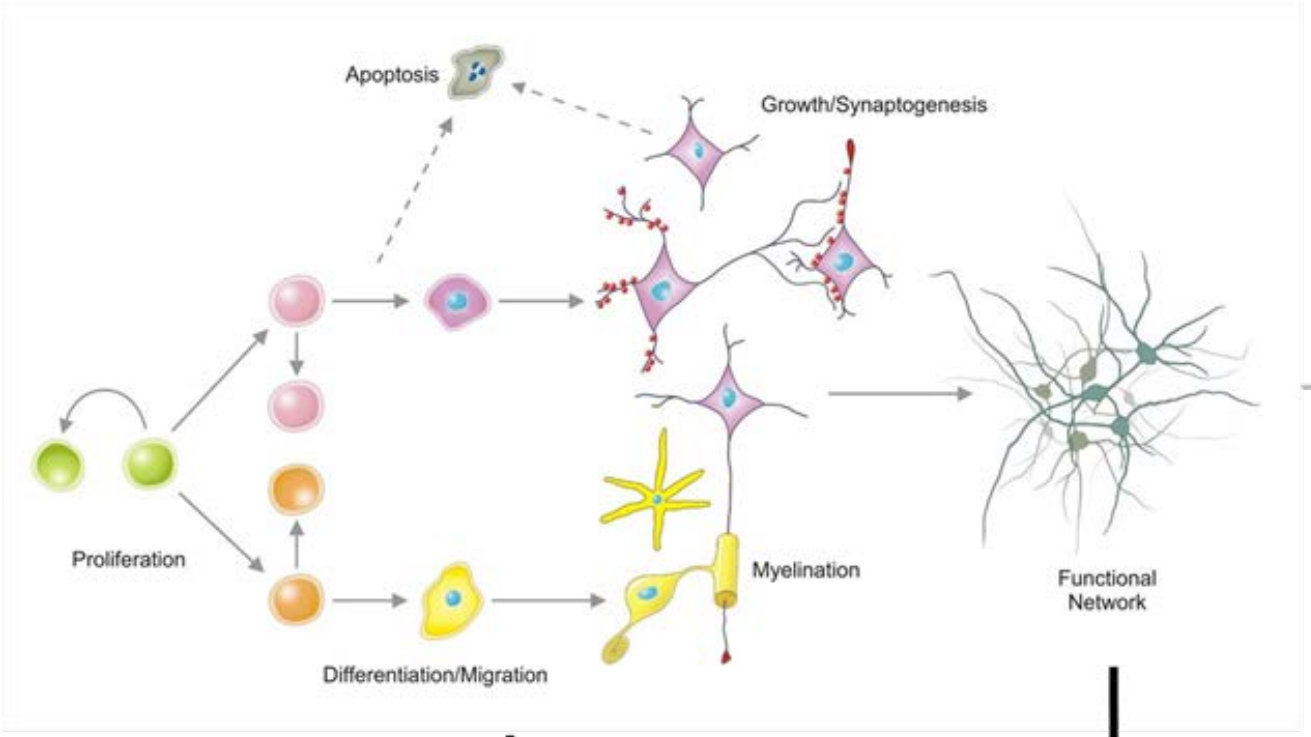
Shift to Targeted Testing

- OPP has shifted its testing focus from DNT guideline study to more targeted testing based on commonly accepted modes of action (MOA)
  - OPs and NMCs: comparative cholinesterase assays (CCA) to evaluate AChE inhibition across lifestages
  - Pyrethroids: evaluate lifestage susceptibility using *in vitro* studies and physiologically based pharmacokinetic (PBPK) modeling based on the known MOA, which involves interaction with voltage-gated sodium channels leading to neurotoxicity
  - Thyroid toxicants: comparative thyroid assay (CTA)

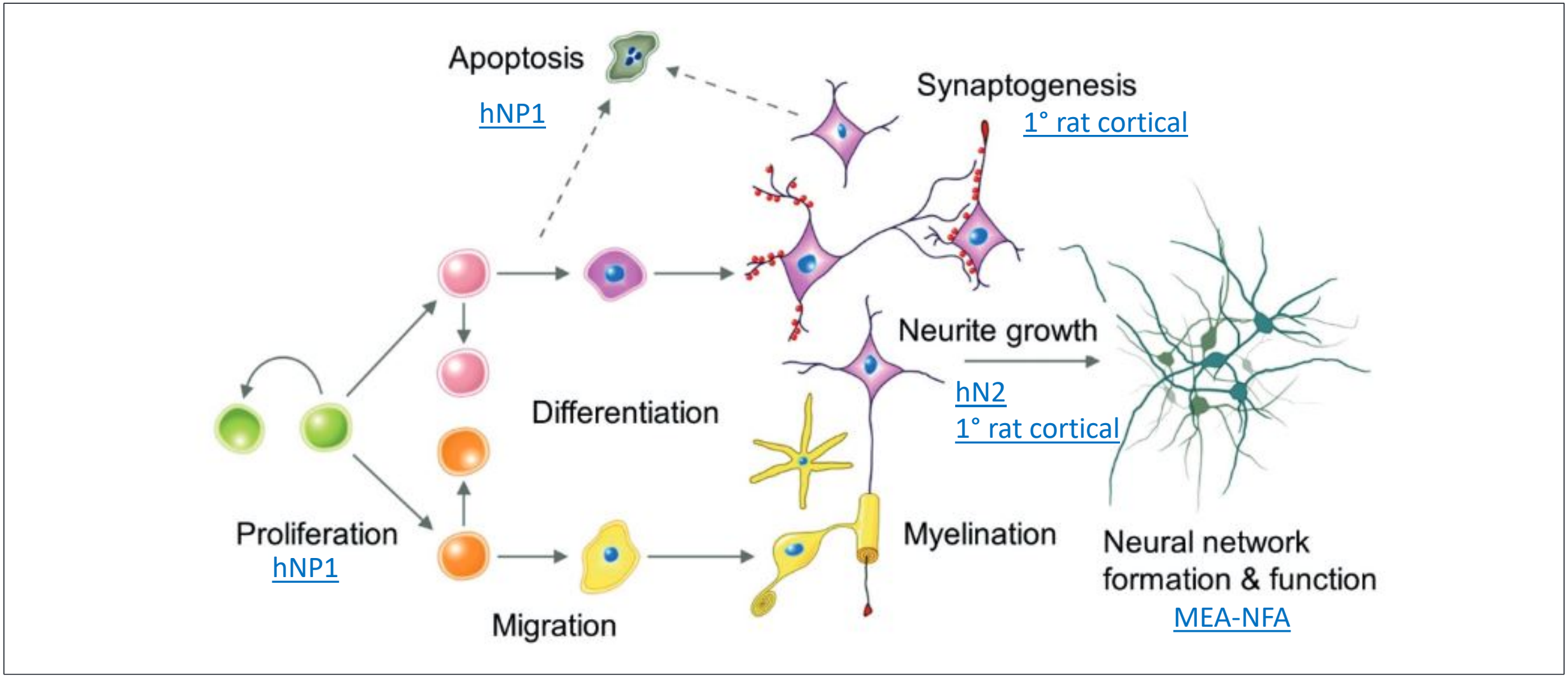
Development of NAMs to Evaluate DNT

- Inclusion of NAMs that take advantage of newer technologies is the logical next step in EPA's efforts to implement more human relevant and efficient approaches
- Office of Research and Development (ORD) researchers collaborating with investigators funded by European Food Safety Authority (EFSA) to evaluate a battery of *in vitro* DNT assays that assess critical processes of neurodevelopment

EPA-ORD DNT NAMs



EPA-ORD DNT NAMs



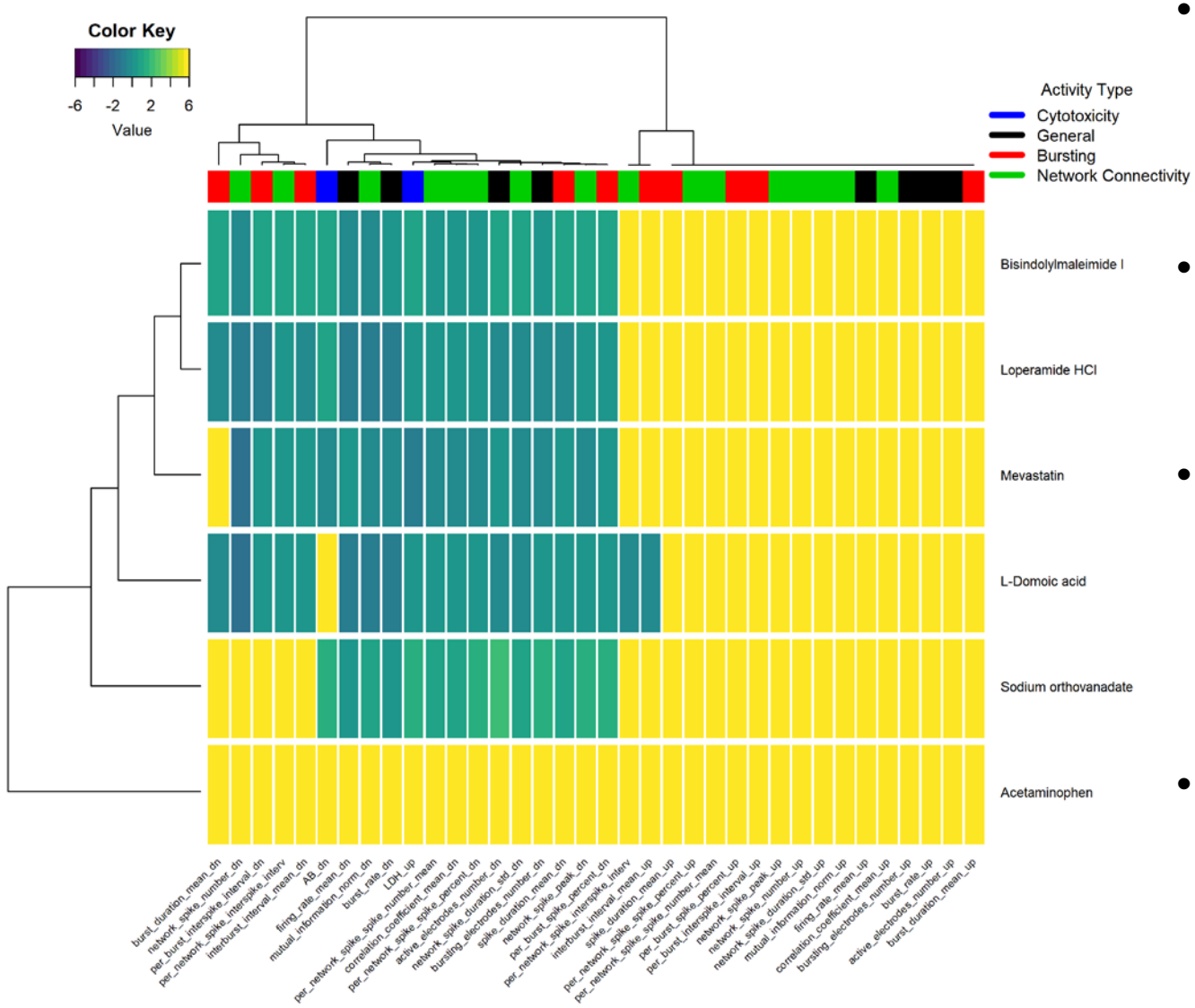
Adapted from Aschner et al., 2016

Reproducibility and Performance of MEA NFA and HCI Assays

- CV of the vehicle control wells in these assays are generally less than 20% and suggest that these assays are reproducible
  - MEA NFA: average CV was 16% across all 18 assay components, with a range of 6.57-25.2%
  - HCI assays: average CV was 8.74% across all 21 assay components, with a range of 3.66-16.6%
- Repeat screening of 21 chemicals in MEA NFA resulted in:

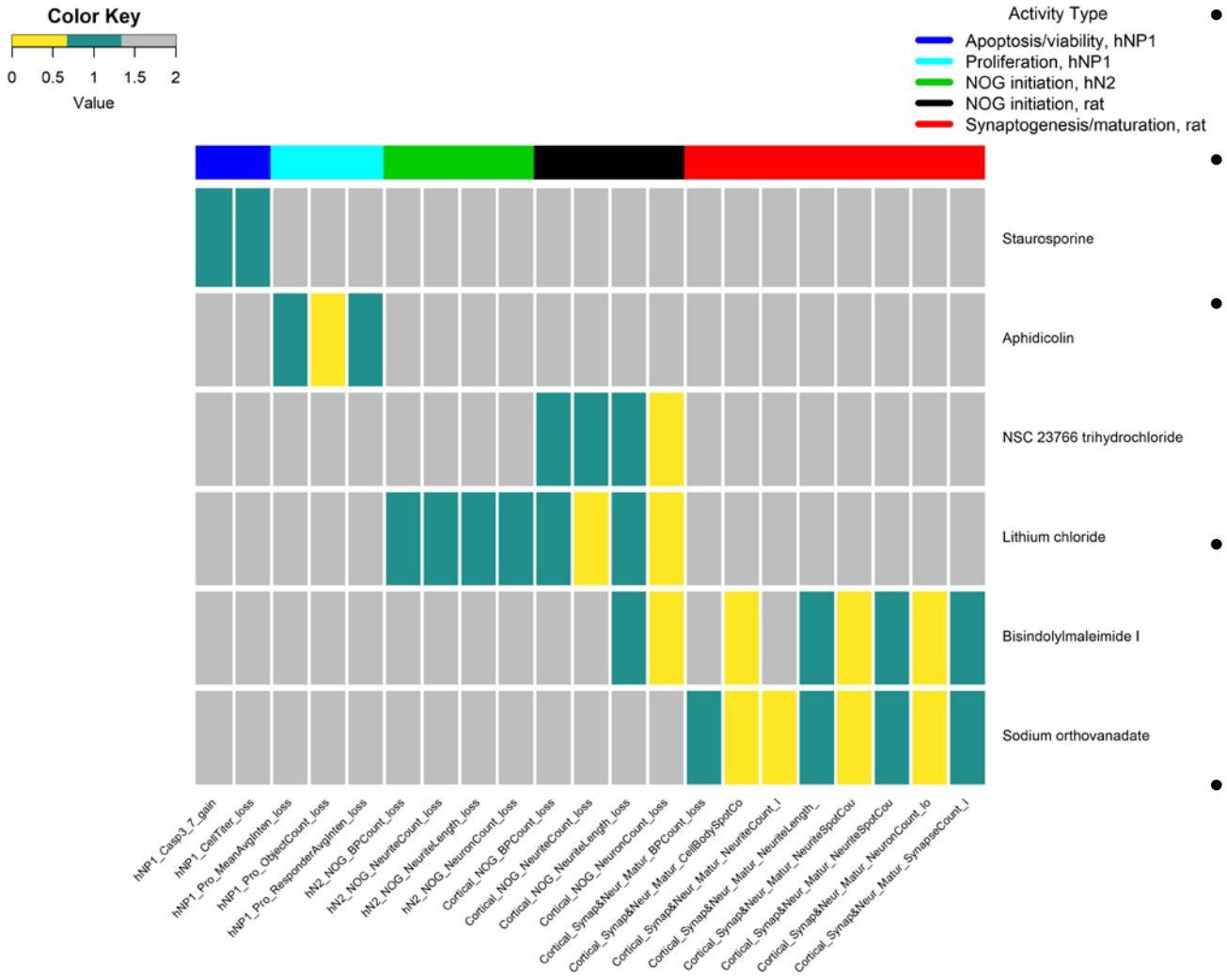
Repeat (+)	Repeat (-)/ Equivocal (≤ 3 assay endpoints positive)	Mixed positive and negative	Qualitative Concordance	Quantitative Concordance
10	5	6	15/21 = 71%	standard deviation in log10-AC50 values (in micromolar units) was less than 0.5 log10-micromolar, except for 2,2',4,4'-tetrabromodiphenyl ether, where there was more uncertainty in the potency values (average standard deviation across all AEIDs for this chemical was 1.71 log10-micromolar)

Performance Controls – MEA NFA



- Biology: 5 positive controls: loperamide hydrochloride, bisindolylmaleimide I, L-domoic acid, mevastatin, and sodium orthovanadate; affect neurite outgrowth and synaptogenesis *in vitro*
- Directionality: Decreased general, bursting, and network connectivity; No controls consistently increased these signals (unlike acute MEA)
- Reproducibility: Median Z' for all the assay performance controls (0.55 to 0.8) indicates reproducible, robust results; Larger effect sizes corresponded to higher Z' values; bisindolylmaleimide I and L-domoic acid were generally the most efficacious and reproducible controls
- Selectivity: Cytotoxicity and activity occurred within a narrow concentration range (within one order of magnitude) for positive controls.

Performance Controls – HCI Assay



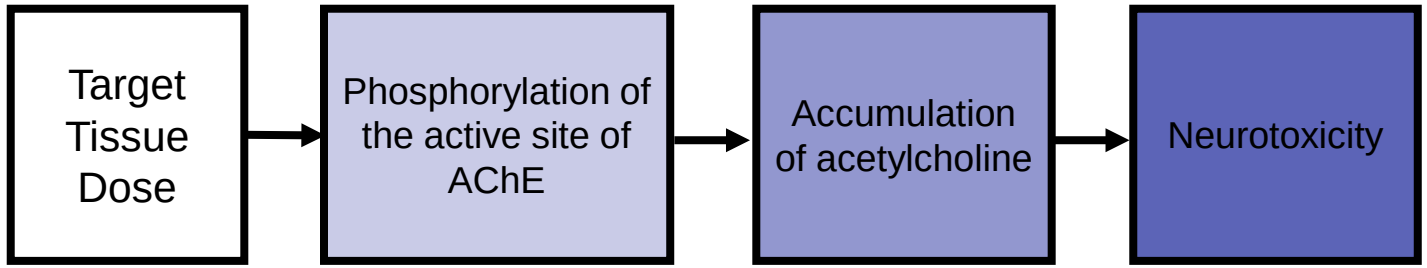
- Apoptosis/viability: *staurosporine*: robust Z' values >0.75 and large effect sizes distinguishable from baseline.
- Proliferation: *aphidicolin*: small effect sizes with more variability (low SN and Z').
- Neurite outgrowth (NOG): *lithium chloride* (in hN2): affected # of branch points and neurites, with smaller, less reproducible effect sizes for neurite length; (in rat cortical): most robustly affects neurite length.
- Rat cortical NOG: *Bis I:* had moderate effects on neurite length (in the absence of cytotoxic effects on neurite count); *NSC 23766* very robustly affected branch point and neurite count as well as neurite length, with Z' all ≤ 0.5 and large SSMD values.
- Neurite maturation and synaptogenesis: *Bis I*: moderate effects sizes for branch point, neurite, and synapse count as well as neurite length, with concomitantly moderate Z' scores in the 0.3 range. *Sodium orthovanadate:* small effect sizes with more variability, and consequently demonstrated very low Z' values.

Case Study: OP Pesticides

- FIFRA requires EPA to review each registered pesticide at least every 15 years for Registration Review
  - Under FQPA, EPA must consider aggregate risk, cumulative exposure, and potential for susceptibility of children to pesticides
- Current Registration Review of OPs due for completion by October 1, 2022
  - 22 OPs undergoing Registration Review
- Given large amount of data available for OPs and widespread acceptance of neurotoxic MOA, OPs identified as case study for the development of a battery of NAMs to evaluate DNT

Case Study: OP Pesticides

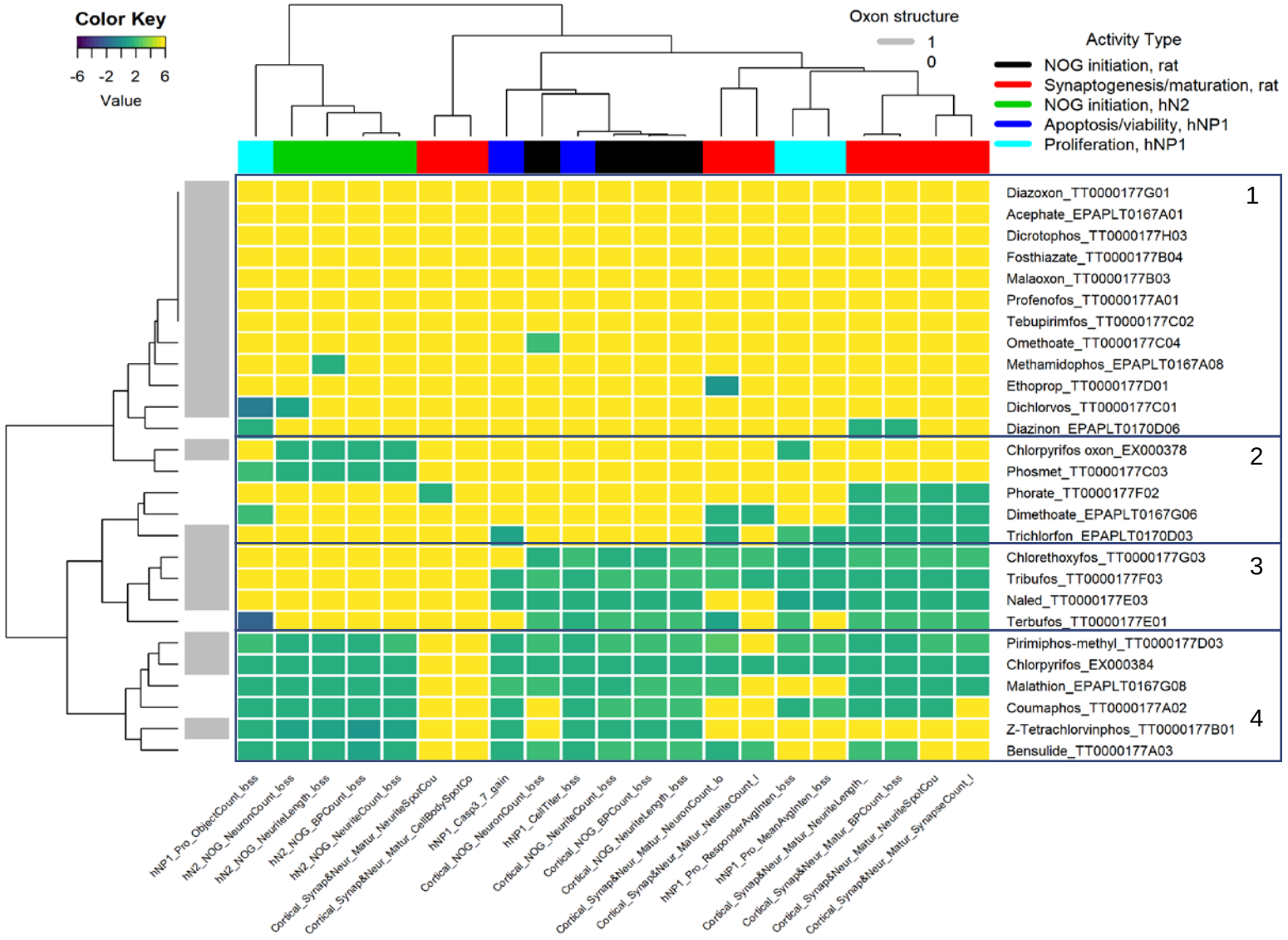
- OPs are a class of insecticides with numerous uses including agricultural applications and mosquito control
- In 1999, EPA determined OPs form a common mechanism group
  - Shared ability to irreversibly bind to and phosphorylate AChE in central and peripheral nervous systems leading to accumulation of acetylcholine and ultimately neurotoxicity
- Acetylcholinesterase (AChE) inhibition used as basis of PODs for risk assessment
- Some OPs require activation to an oxon metabolite, which is the active moiety



Case Study: OP Pesticides

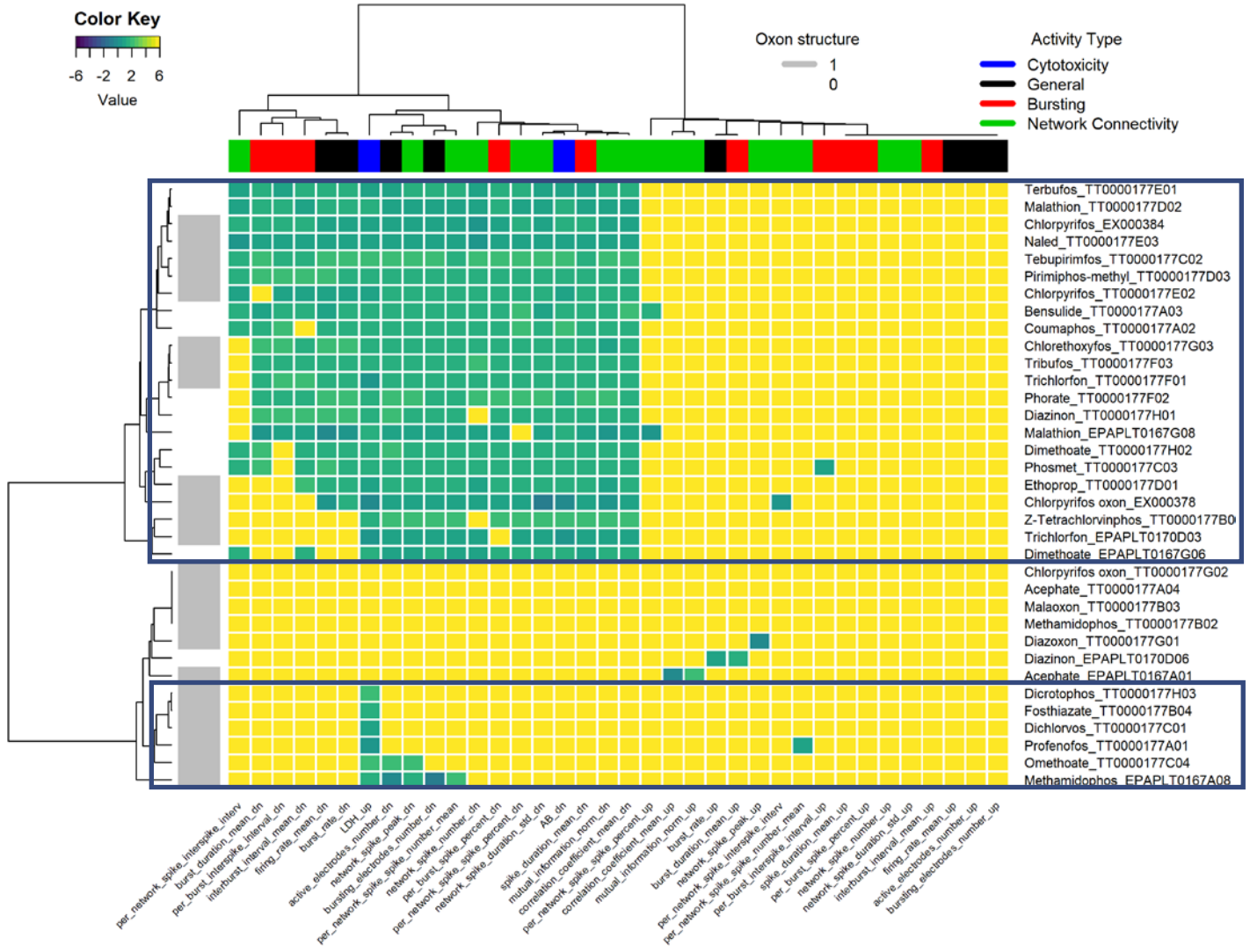
- Newer lines of research on OPs have raised uncertainty with respect to the potential DNT effects in fetuses and children
  - Interpretation has been challenging due to conflicting results across studies
  - MOAs have not been established for neurodevelopmental outcomes
- Series of FIFRA SAP meetings held in recent years for chlorpyrifos regarding the use of epidemiological data to inform chlorpyrifos human health risk assessment and relevance to other OPs
  - SAP reports have provided numerous recommendations for additional research and sometimes conflicting advice for how EPA should consider (or not consider) the epidemiology data in conducting human health risk assessments for OPs
- Data from *in vitro* assays may be considered as part of an overall WOE to determine the DNT potential for individual OPs and used to inform the FQPA Safety Factors (FQPA SF) in the future, if appropriate

HCI Results



- Many OPs were positive in HCI cellular event assays for neural progenitor cell proliferation, apoptosis, neurite outgrowth and/or synaptogenesis
- Some OPs were inactive across all assays in the HCI suite

MEA NFA Results



- Top active cluster of OPs contains oxon and non-oxon structures.
  - Like assay performance controls, these OPs appear to generally decrease all activity types and most assay endpoints
- Cytotoxicity and activity occur within a narrow concentration range
- Bottom cluster with minimal actives appears somewhat driven by cytotoxicity in the LDH assay
- While not all OPs are active in the MEA NFA, those that are active appear to behave much like the assay performance controls that inhibit NOG and/or synaptogenesis

Comparison of MEA-NFA and HCI

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			

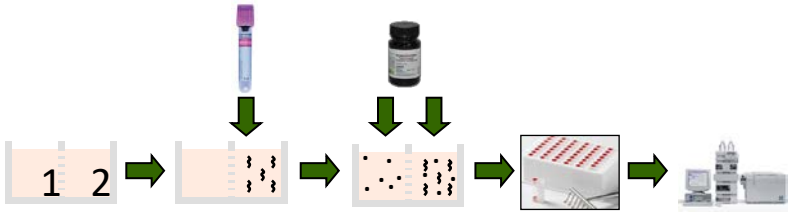
DTXSID	Chemical	Neg	Equiv	Pos	1	2	3	4
DTXSID4032459	Phorate			X		X		
DTXSID5024261	Phosmet			X		X		
DTXSID0024266	Pirimiphos-methyl			X				X
DTXSID3032464	Profenofos		X		X			
DTXSID1032482	Tebupirimfos			X	X			
DTXSID2022254	Terbufos			X			X	
DTXSID1024174	Tribufos			X			X	
DTXSID0021389	Trichlorfon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

High Throughput Toxicokinetics (HTTK)

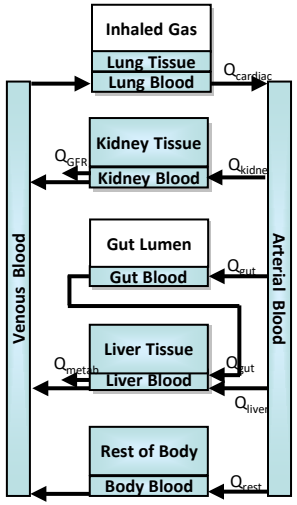
in vitro data

Hepatic clearance from suspended hepatocytes



Plasma protein binding

Generic toxicokinetic models



= *httk*

Some high-level assumptions:

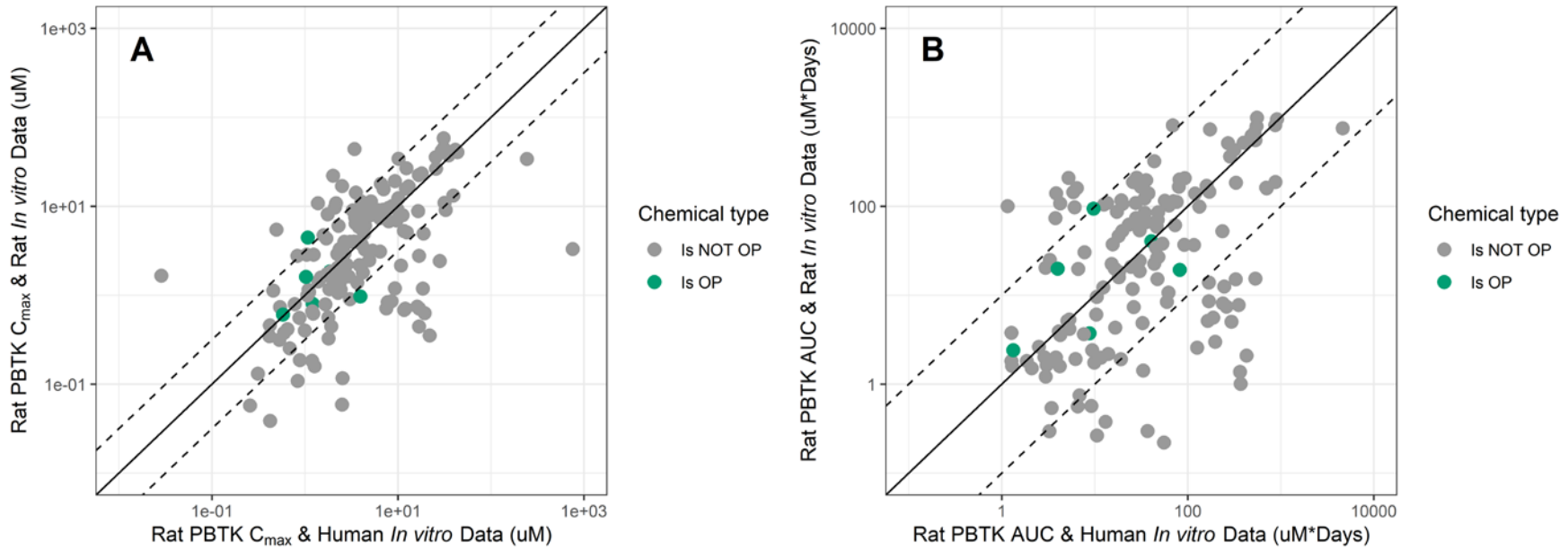
(1) bioactive nominal *in vitro* assay concentration ~ *in vivo* plasma concentration that would correspond to a similar effect;

(2) plasma concentration can be approximated by steady-state kinetics; and,

(3) external exposures (in mg/kg/day units) that may have resulted in that plasma concentration can be constructed using estimates of species-specific physiology and Phase I and Phase II enzyme-driven hepatic clearance.

Slide modified from John Wambaugh

Use of Human HTTK Data for Rat Model

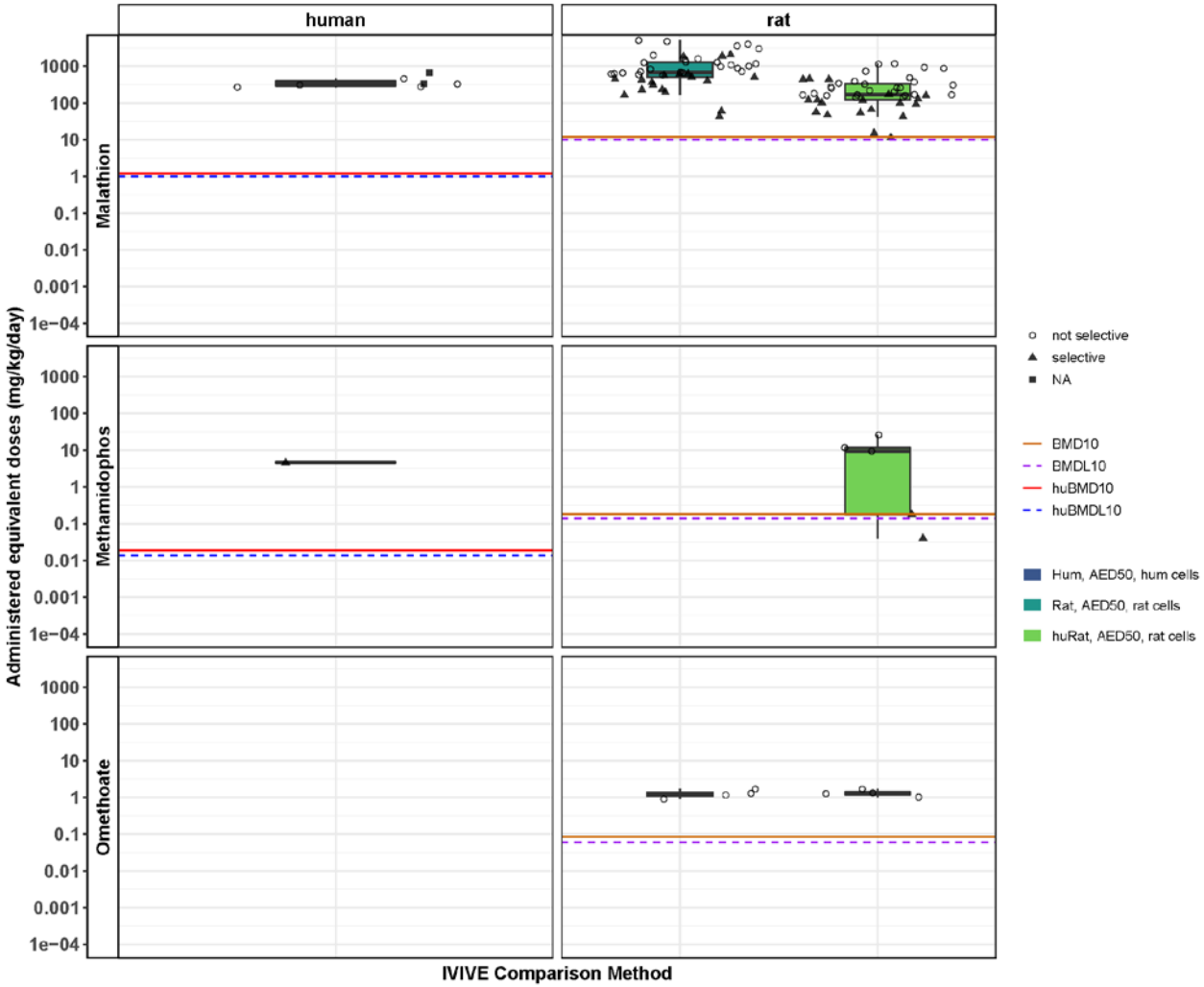


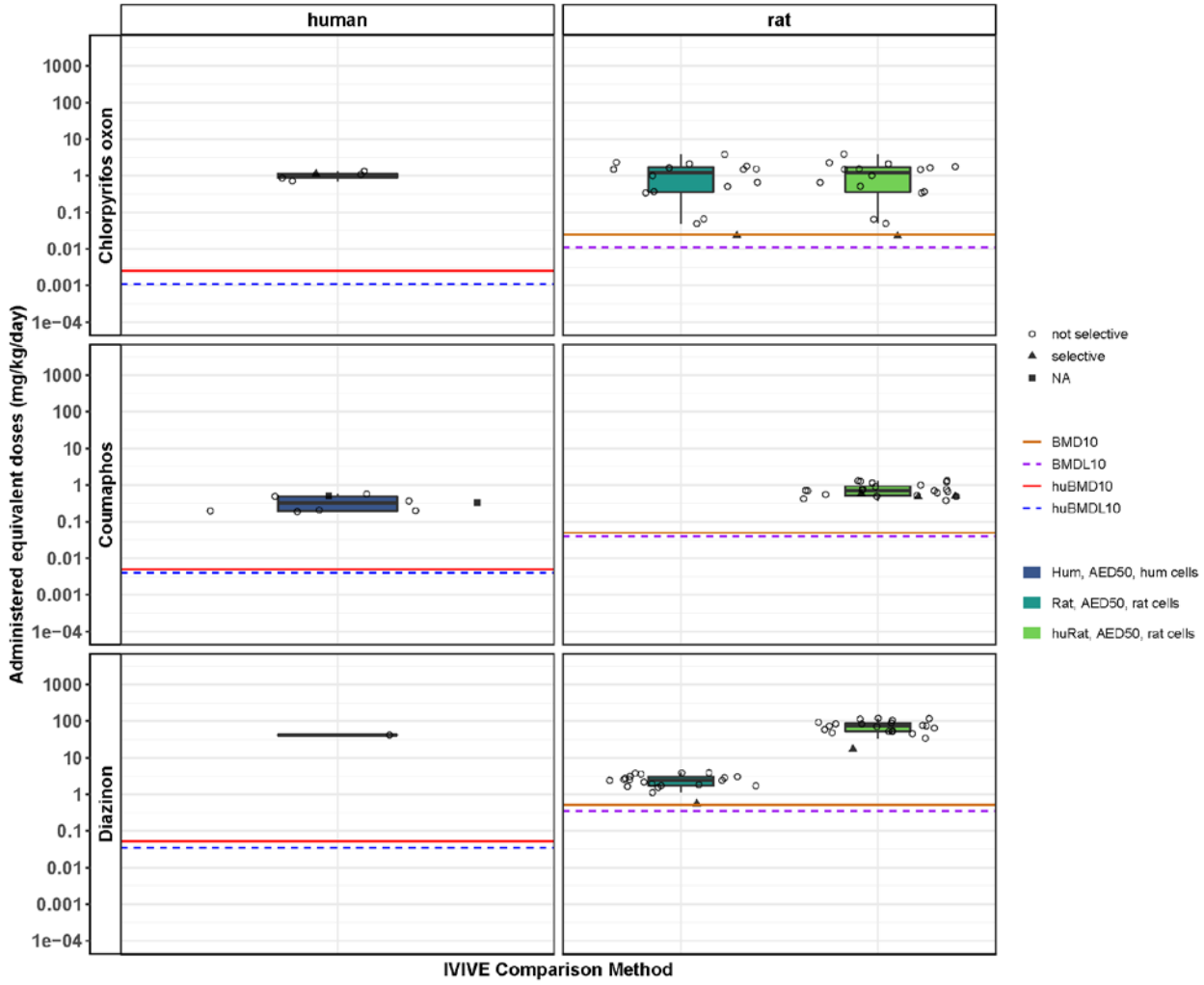
- In the absence of hepatic clearance values from rat hepatocytes, rat liver microsomes, or rat liver Phase I enzymes, would the use of human hepatocyte-derived hepatic clearance values be a reasonable substitute?
- In addition to comparing rat-derived administered equivalent dose (AED) values to benchmark dose (BMD) values from rat studies, compared AED values from the “humanized-rat” or the huRat, which used human HTTK data in a model parameterized with rat physiology, to BMD values from rat studies

General Approach to AED50 Estimation

- **Human comparisons:**
  - 9/57 total assay endpoints in the DNT-NAM battery evaluate neural cell proliferation, apoptosis and viability, and neurite outgrowth initiation using human-derived cells
  - human-derived HTTK data and a human-parameterized 3 compartment steady state (3compss) model
  - Compared to BMD10/BMDL10 values from rat divided by an uncertainty factor of 10 (default interspecies uncertainty factor applied in risk assessments to account for extrapolation from laboratory animals to humans)
  - **Only 17 OP chemicals had positive values in the human assays, and of these 14 had sufficient HTTK data and modeling to calculate human AED_{50} values with the 3 compss model**
- **Rat/huRat comparisons:**
  - 48 of the 57 total assay endpoints in the DNT-NAM battery that evaluate neuronal network formation and function, neurite outgrowth initiation, and neurite maturation and synaptogenesis were evaluated using rat primary cortical cells
  - rat-derived (or for huRat, human derived intrinsic clearance data) and a rat-parameterized 3 compss model
  - Compared to BMD10/BMDL10 values from rat
  - **23/27 OP chemicals have enough data and modeling available to derive huRat AED_{50} values**
  - **9 OP chemicals had enough data and modeling available to derive rat AED_{50} values**
- Three OP chemicals (chlorethoxyfos, naled, Z-tetrachlorvinphos) excluded from the IVIVE comparisons due to chemical instability in the matrices used for HTTK assays (unpublished, Wetmore 2020)
- Malaoxon was completely negative in all assay endpoints

Example AED/BMD Comparisons



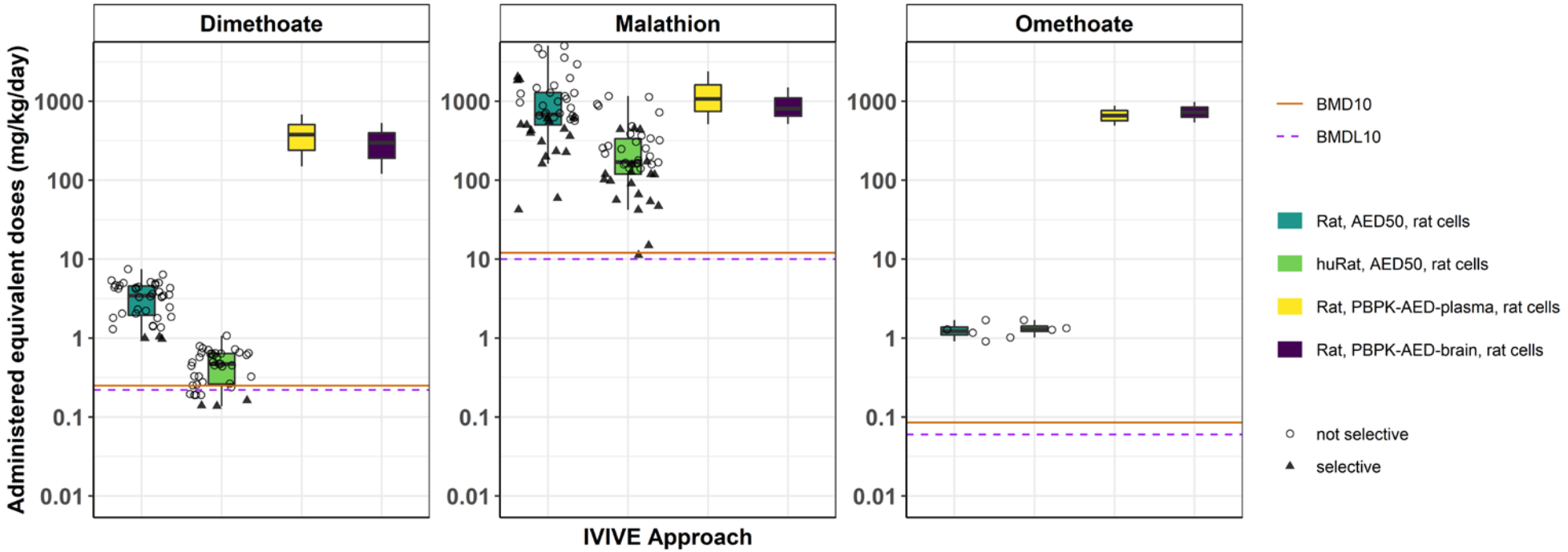


AED to BMD/BMDL Comparison

- Some rat and huRat AED_{50} values approached the *in vivo* rat BMD10 and BMDL10 thresholds identified using *in vivo* rat studies of AChE
- The human IVIVE comparison was more constrained because fewer OP chemicals had positive responses in this smaller subset of the DNT-NAM assay set
- The human AED_{50} values and huRat AED_{50} values were typically similar; both of these sets of values use the human HTTK data to inform human and rat models, respectively.
- Chemical-dependent differences between the rat and huRat AED_{50} values are apparent when both are available
  - For some chemicals (chlorpyrifos oxon, ethoprop, malathion, omethoate) the values are very similar
  - May be as much as 1 log10 order of magnitude separation between the median AED_{50} values for other chemicals (bensulide, chlorpyrifos, diazinon, diazoxon, dimethoate)
  - No uniform direction to observed differences, but these differences are expected and consistent with the impact of using human or rat HTTK data to inform a rat physiology-based model

Overall, these comparisons suggest that the estimated doses required to achieve plasma concentrations (in the median individual in the general population) that demonstrate *in vitro* bioactivity relevant to DNT are higher than or in some cases approaching/equal to the doses that have been associated with significant changes in AChE activity in rats.

PBPK Modeling



- Dimethoate and omethoate: PBPK-AED values using plasma and brain area under the curve (AUC) were more than two orders of magnitude greater than the HTTK-derived AEDs
- Malathion, the PBPK-AED values were similar to the range of HTTK-derived AED_{50} values for rat

Coverage of Important Processes

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

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Towards regulatory DNT testing: Alternative methods

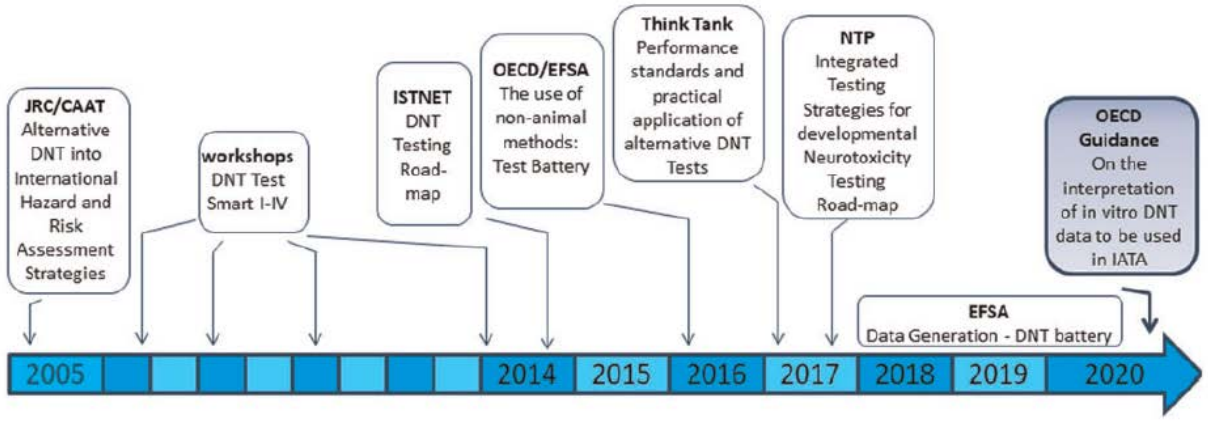
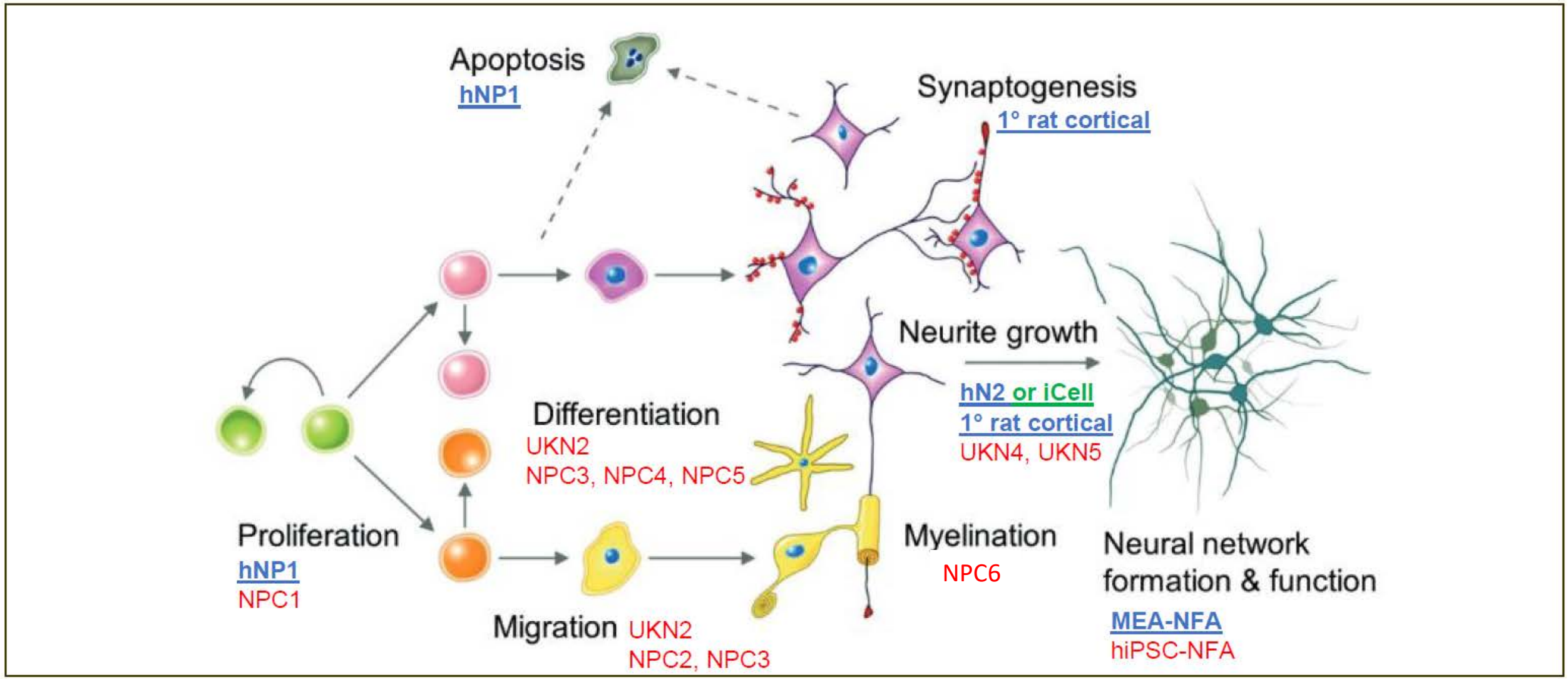


Figure 1. Timeline of efforts to develop and implement new alternative methods for developmental neurotoxicity.

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	~~Cell 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)



Adapted from Aschner et al., 2016

Testing in EPA-ORD and EFSA-Sponsored Assays

- "Chemical Library" developed to test common set of compounds
- Identified ~136 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
  - Putative negative compounds
  - Modulators of developmental pathways
- These compounds will be tested in the 12 different DNT assays
- ToxCast has supplied most of these compounds
- Compounds will be tested by EPA, University of Konstanz and University of Duesseldorf in a variety of in vitro assays

FIFRA SAP Meeting – September 2020

- Solicited comment from Scientific Advisory Panel (SAP) on robustness of *in vitro* assays developed by ORD for evaluating DNT endpoints and the ability of the currently available battery of assays (ORD and EFSA-sponsored) to cover critical processes in neurodevelopment
- Also solicited comment on comparison of administered equivalent doses (AEDs) to BMD values based on AChE inhibition to predict relative sensitivity
- Report published within 90 days of the meeting

https://www.epa.gov/sap/fifra-scientific-advisory-panel-meetings

Conclusions

- ORD assays recapitulate key cellular events and processes relevant to DNT, as demonstrated through the use of appropriate assay performance controls
- ORD assays demonstrate reproducibility in terms of positive responses and potency of these responses
- 27 OP chemicals in this set are differentially active in the MEA-NFA and HCI assay suite
- IVIVE approaches for *in vitro* bioactivity observed in ORD assays result in AED_{50} values that are greater than or in some cases approximate doses that inhibit AChE *in vivo*
- OP data from these assays will be considered in combination with the results of EFSA-sponsored assays as part of an overall weight of evidence evaluation of the DNT potential for individual OPs

EPA NAM Workplan

- Develop NAMs that fill critical information gaps
  - Overcome challenges/limitations with *in vivo* DNT guideline study
  - Fit-for-purpose use of data and information

- Establish scientific confidence and demonstrate application
  - Measure biological processes critical for neurodevelopment using model systems representative of in vivo neurobiology
  - Evaluation of reproducibility and performance
  - OP case study
  - Research products (e.g., publications)

- Engage and communicate with stakeholders
  - September 2020 Scientific Advisory Panel
  - International collaboration

https://www.epa.gov/chemical-research/new-approach-methods-work-plan

Thank you!