Examples of How Data from in vitro Developmental Neurotoxicity Assays Are Being Used to Make Decisions About Chemicals

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

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International Efforts on DNT NAMs

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Forum

FORUM

International Regulatory and Scientific Effort for Improved Developmental Neurotoxicity Testing

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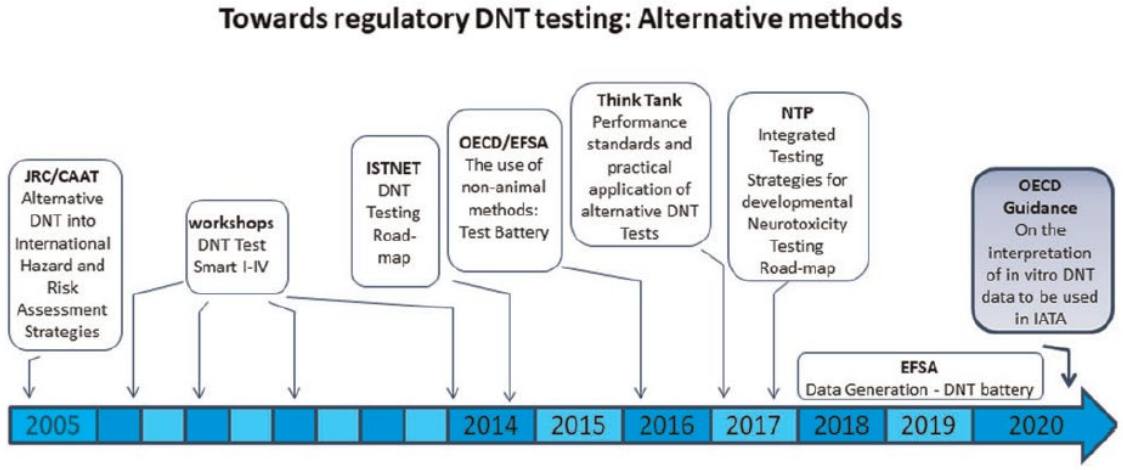


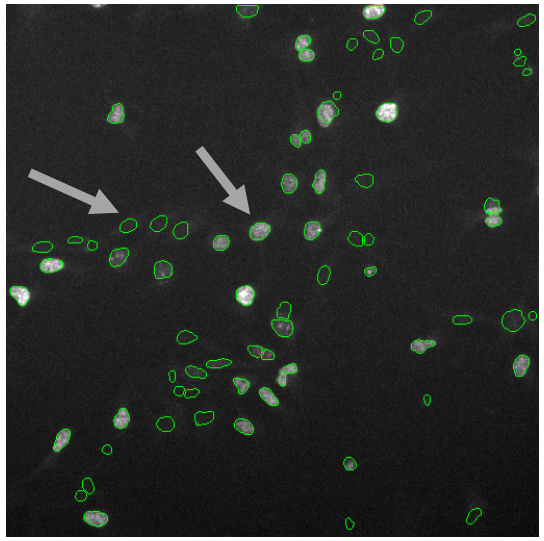
Figure 1. Timeline of efforts to develop and implement new alternative methods for developmental neurotoxicity.

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

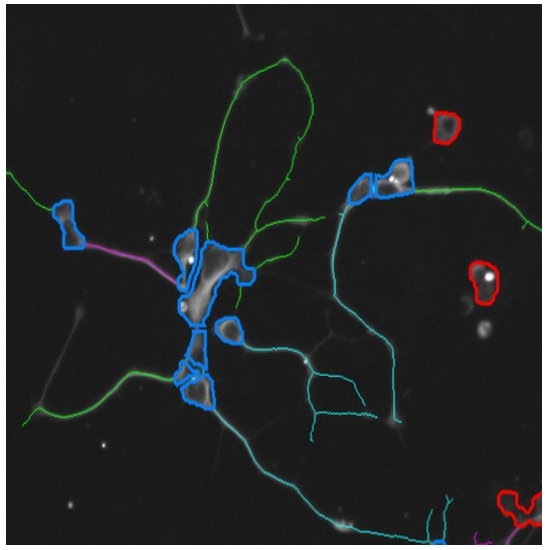
Process	Assays	References
Proliferation	hNP1	Harrill *et al.* (2018)
	NPC1	Baumann *et al.* (2016) and Barenys *et al.* (2017)
	UKN1	Balmer *et al.* (2012)
Apoptosis	hNP1	Harrill *et al.* (2018)
Migration	NPC2	Baumann *et al.* (2016) and Barenys *et al.* (2017)
	UKN2	Nyffeler *et al.* (2017)
Neuron differentiation	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)

EPA DNT NAM Assays

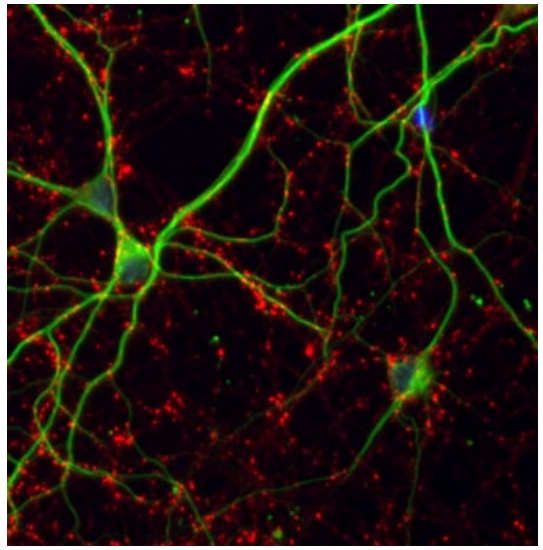
Proliferation



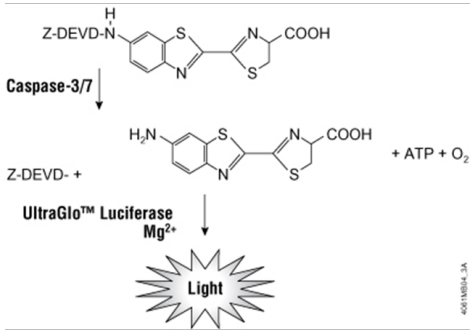
Neurite Outgrowth



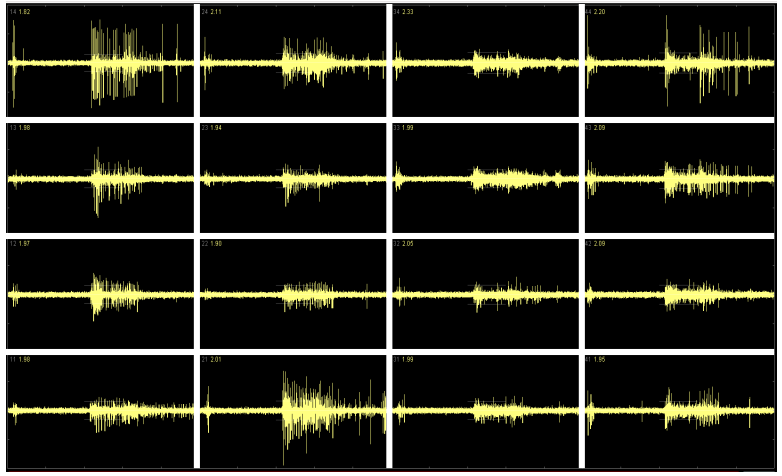
Synaptogenesis



Apoptosis

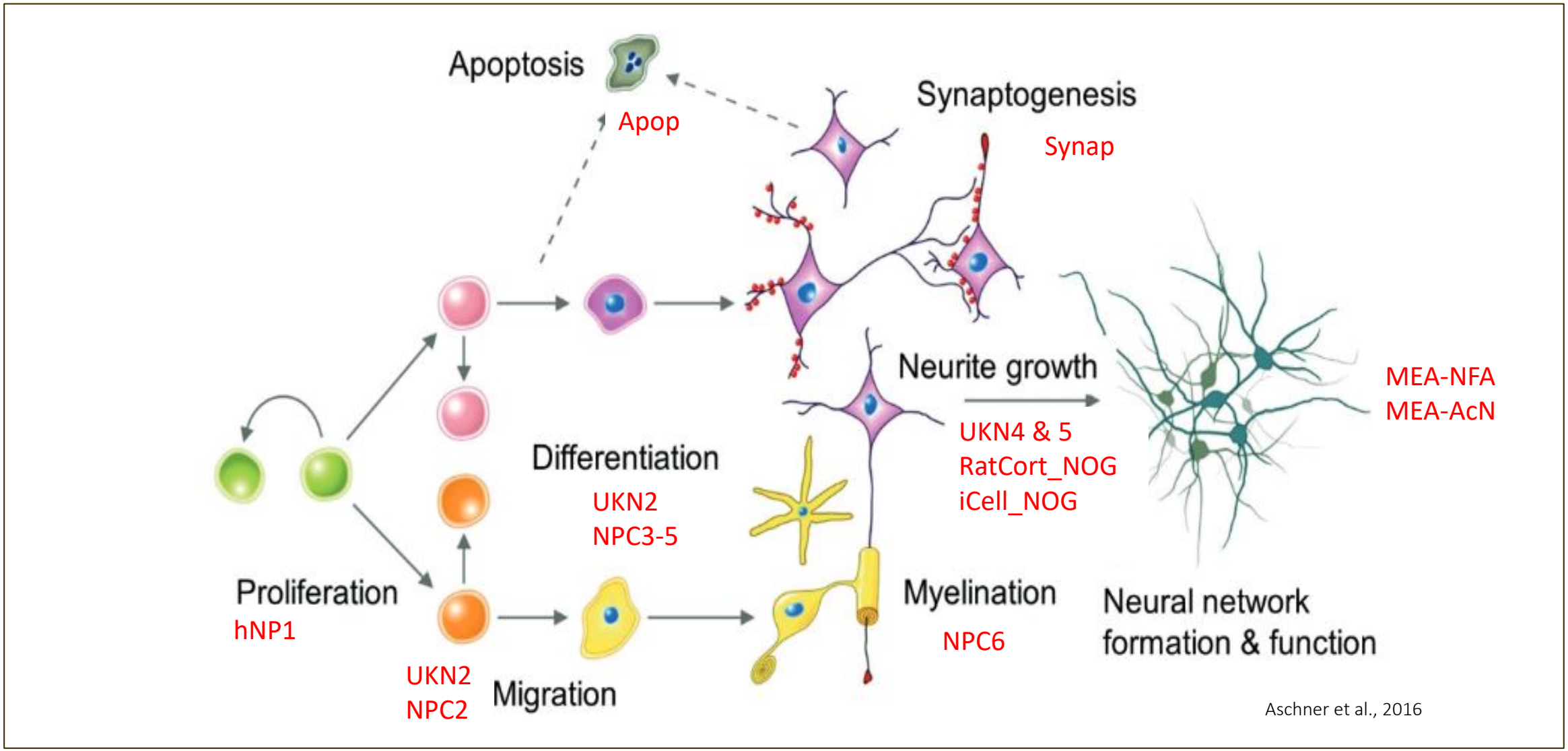


luminescence



Acute Network Function (MEA-AcN) and Network Formation Assay (NFA)

DNT NAMs Coverage of Neurodevelopmental Processes



Aschner et al., 2016

Examples of the use of DNT NAMs at EPA

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

II. **Structure-activity relationships**
   - Apply DNT NAMS to evaluate structurally similar chemicals when one of the chemicals has known effects in a Guideline DNT study
      - Example with compound X and structurally similar analogs
         - DNT Guideline exists for X, should it be required for the analogs?

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

Example #1: Screening Level Information for PFAS Compounds

Problem: Perfluoroalkyl substances have recently been identified as environmental contaminants with significant human exposure. Little toxicological information is available for these compounds.

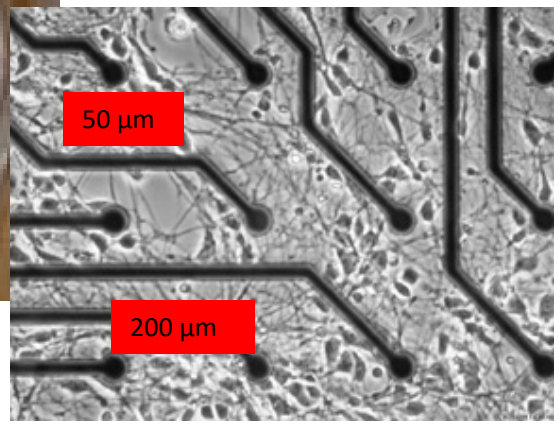
- Structurally diverse
- With the exception of a few specific congeners, little toxicological information
- Evidence of DNT is ambiguous,
  - epidemiological studies report positive and negative neurodevelopmental effects associated with exposure to PFAS

Assembled a PFAS Chemical Library for Research and Methods Development

- Attempted to procure ~3,000 based on chemical diversity, Agency priorities, and other considerations
- Obtained 480 total unique chemicals
  - 430/480 soluble in DMSO (90%)
  - 54/75 soluble in water (72%) (incl. only 3 DMSO insolubles)
  - Issues with sample stability and volatility
- Subset of PFAS Library for testing:

Hepatotoxicity
Developmental toxicity
Mitochondrial toxicity
Developmental neurotoxicity
Endocrine Disruption
General toxicity

Measuring Network Formation on Microelectrode Arrays



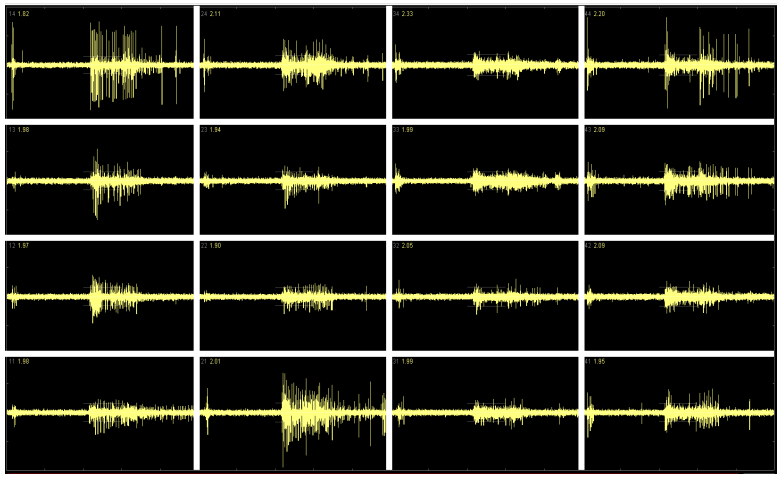
Microelectrode Array (MEA) Recording

- Planar microelectrodes are non-invasive
- Records electrical activity of any tissue type
- Repeated recordings from same sample

The electrical activity recorded by MEAs are the biological underpinnings of EEG recordings.

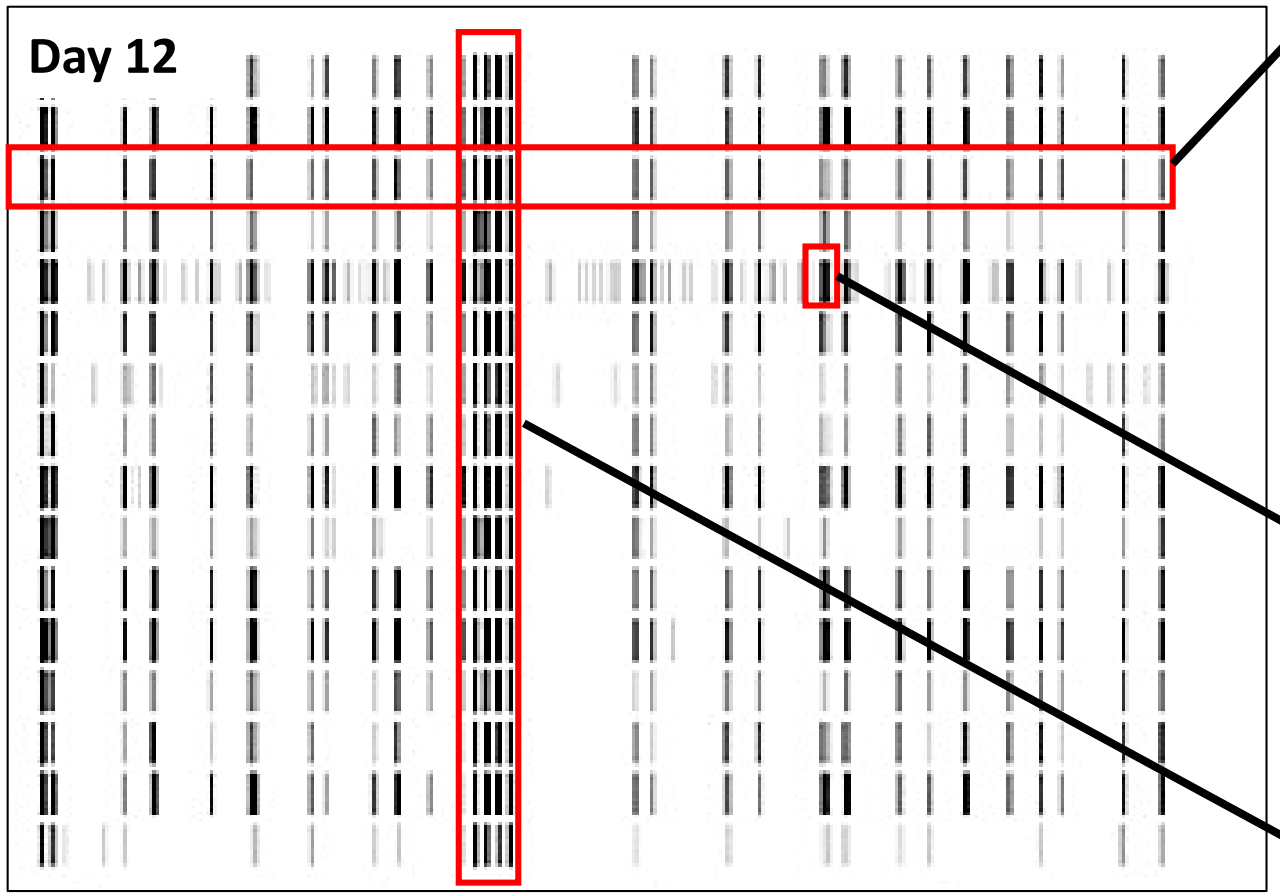
"Brain-on-a-Chip": Complex 2D model

- Rat cortical neural networks
- Contains neurons & glia cells
- Spontaneous activity
- Develops rapidly in vitro
- Follow network development over time
- Integrates activity of multiple processes



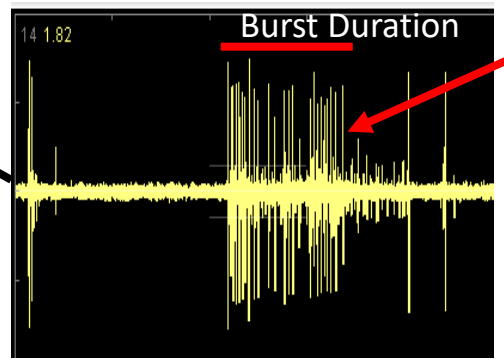
A snapshot in time of neural network activity in one well. Each box represents the electrical activity of neurons on 1 electrode in the array.

MEAs Measure Multiple Characteristics of Network Formation



General Activity- overall rate of firing or bursting; measured on each electrode and averaged across the well.

Bursting Structure- the length and number of events in a burst; measured on each electrode and averaged across the well.



Number of Action Potential "Spikes"/burst

Connectivity- Communication of information across electrodes (Correlation coefficients, Network Spikes, Mutual Information); averaged for the well.

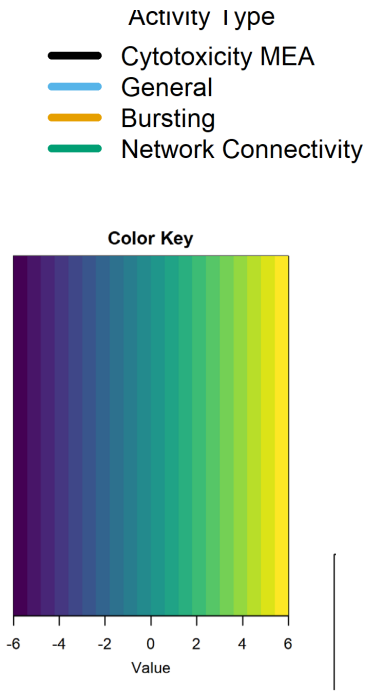
In the Network Formation Assay (NFA), 19 endpoints describing network activity (17) and cytotoxicity (2) are measured over 12 days in vitro. These can increase or decrease following chemical exposure.

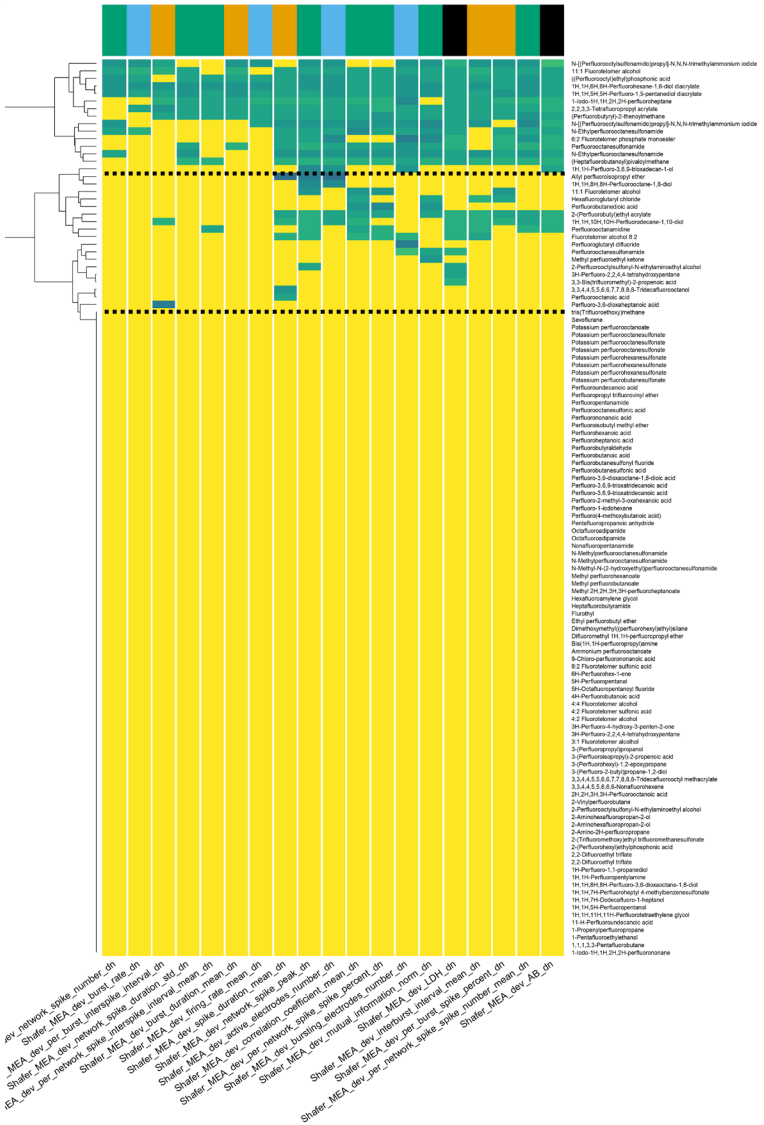
PFAS Compounds Tested for Effects on Network Formation

Test Set of Compounds

- Original PFAS 150
  - 75 tested in concentration-response (0.03-30 µM)
- Re-procured PFAS
  - 131 Tested single-concentration (30 µM)
  - 42 tested in concentration-response (0.03-30 µM)
- 13 compounds tested as biological replicates

Only a fraction of PFAS compounds disrupt network formation

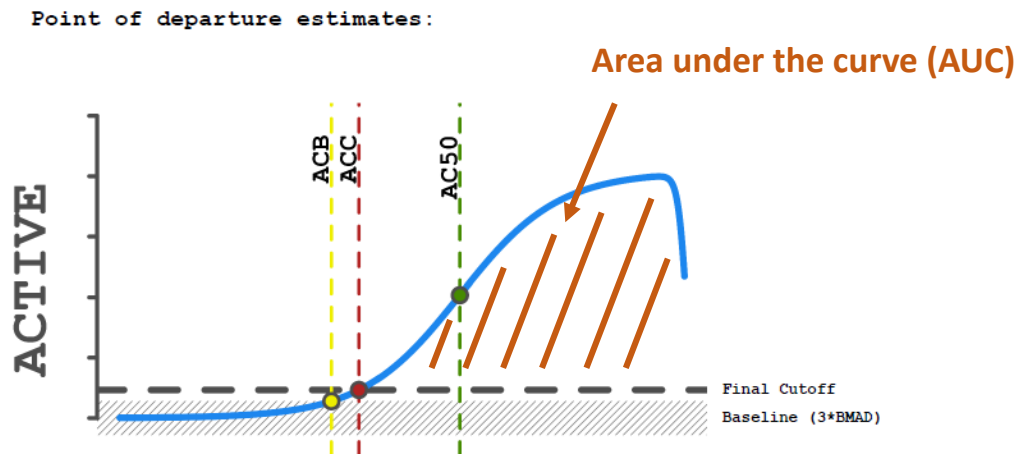


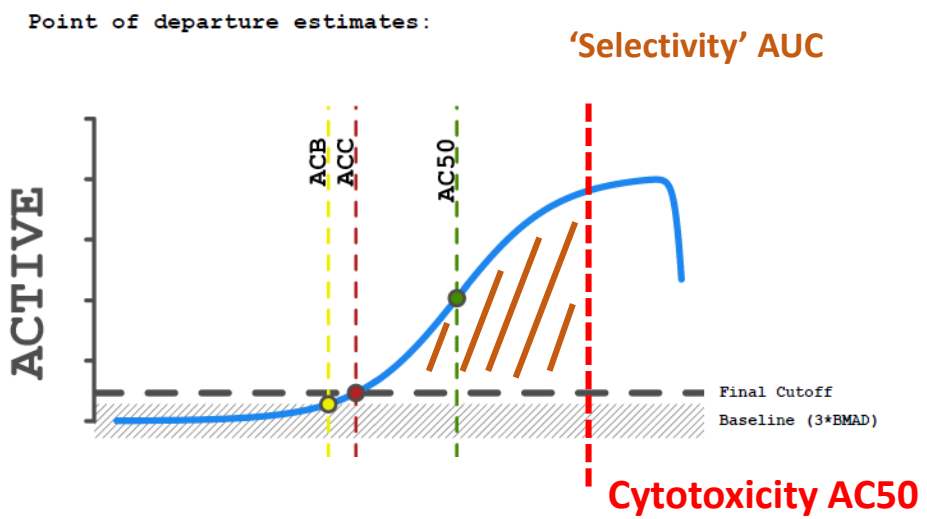


- ~25% of tested compounds were active
- No PFAS compound increased network formation parameters compared to control wells
- Three Groups: 1) “Pan Active” 2) subset of parameters 3) Inactive
- Positive and negative controls gave appropriate responses.
- Replicates gave generally consistent results
- Cytotoxicity was prominent in “Pan Active”

Calculating a ‘selectivity’ metric

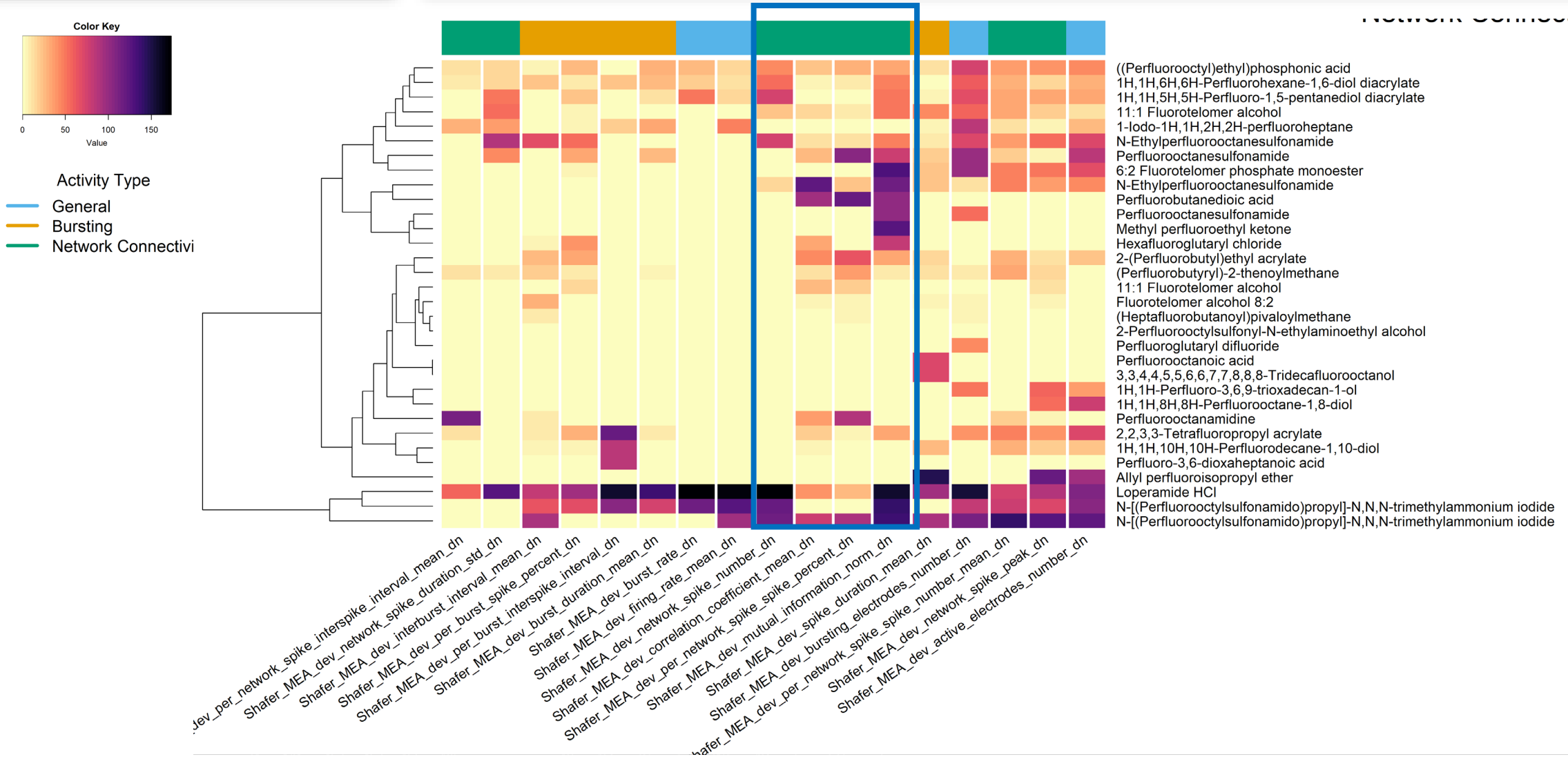
Selectivity: activity at concentrations lower than cytotoxicity.





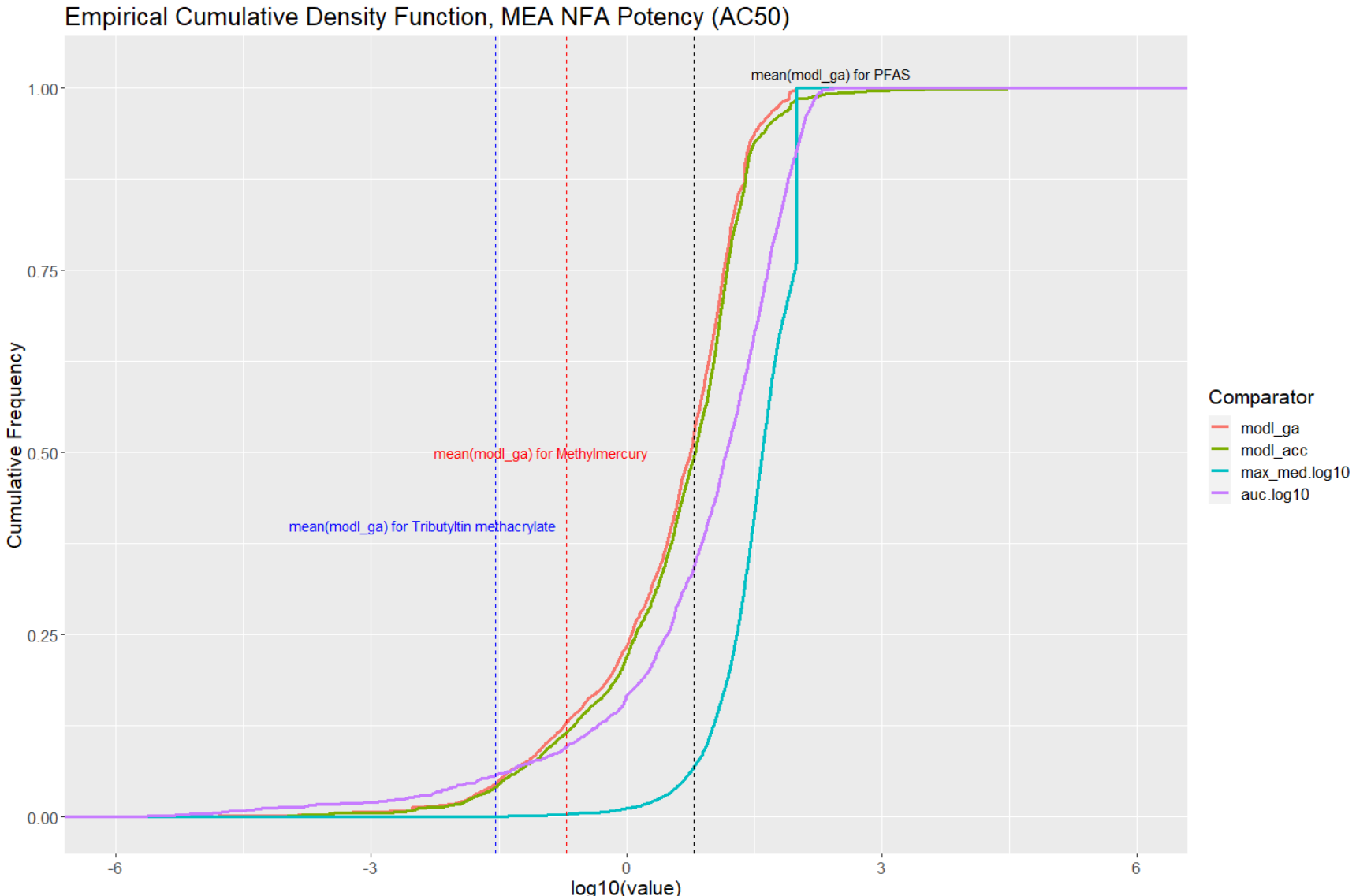
Modified from “The New ToxCast Analysis”, Dayne Filer
https://www.epa.gov/sites/production/files/2015-01/documents/the_new_toxcast_analysis_v2.pdf

Selectivity of PFAS Compounds indicates that General activity and Network Connectivity are Altered more than Bursting



Comparison of PFAS AC_{50} to Other Compounds in the NFA

The potency of active PFAS compounds is near the median of potencies for all compounds tested in the NFA



Summary of PFAS effects in the NFA

The NFA identified PFAS compounds that disrupt the formation of neural networks in vitro

- This identifies compounds with a potential *hazard* for DNT
- Can place the potency of these effects into context with other DNT compounds
- Exposures to these compounds have not been considered.

These data* can be used to make inferences for a broader landscape of PFAS:

- Chemical Category and Read-across approaches for additional hazard identification/characterization.
- Bioactive Dose Level (BDL) Approach (*in vitro* to *in vivo* extrapolation to define administered dose equivalent (ADE) values)

*Data are currently being analyzed across all of the different toxicities evaluated.

Example #2: Using DNT NAMs to evaluate structure-activity relationships

EPA's Office of Pesticide Programs (OPP)

- Registers pesticides for use in the United States.
- Two new compounds (Compound Y, Z)
  - potentially neurotoxic.

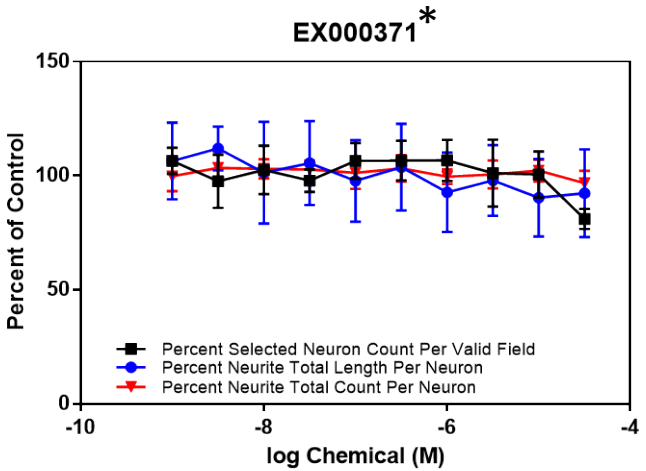
  - A DNT Guideline study existed for "compound X" that was structurally similar to the novel compounds.
    - The DNT Study showed small but statistically significant changes in brain morphology
  - Literature data indicated that compound X caused acute neurotoxicity in vivo and altered network activity in vitro following acute exposure.

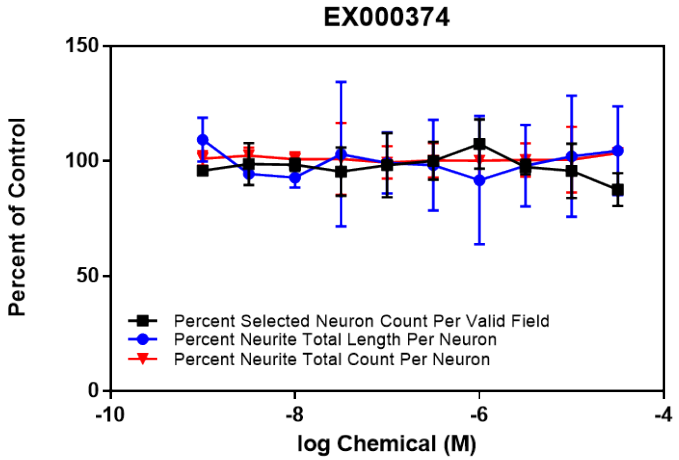
OPP needed to decide whether to request DNT Guideline studies on the new compounds

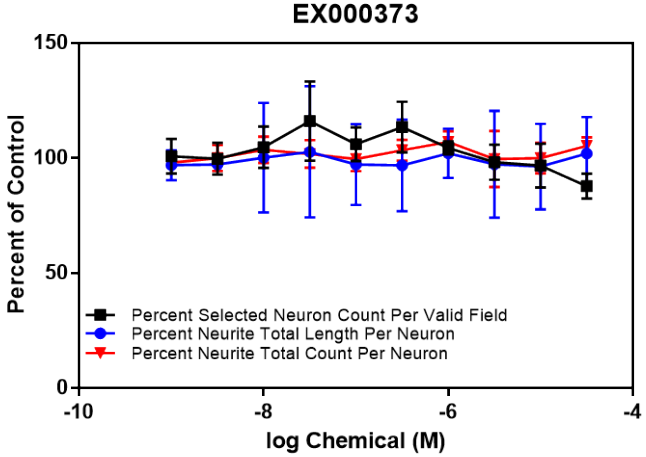
OPP asked EPA's Office of Research and Development to provide data to inform their decision with compounds Y and Z.

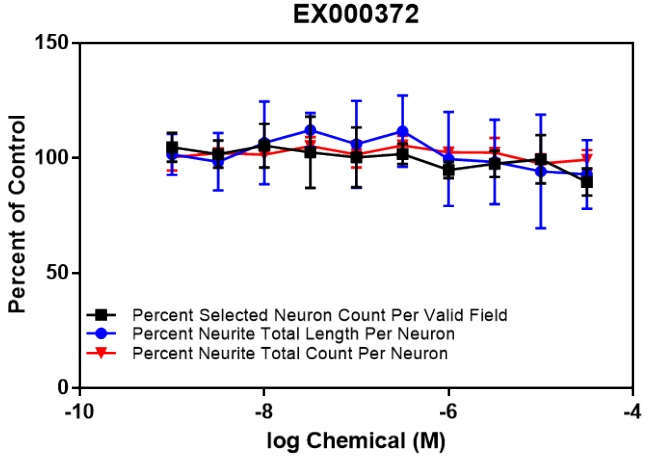
- **Neurite Outgrowth** and **Network Formation** assays were selected based on the of activity of compound X in Guideline Study and in vitro, respectively.
- Compounds X, Y and Z were tested in these assays, along with appropriate controls

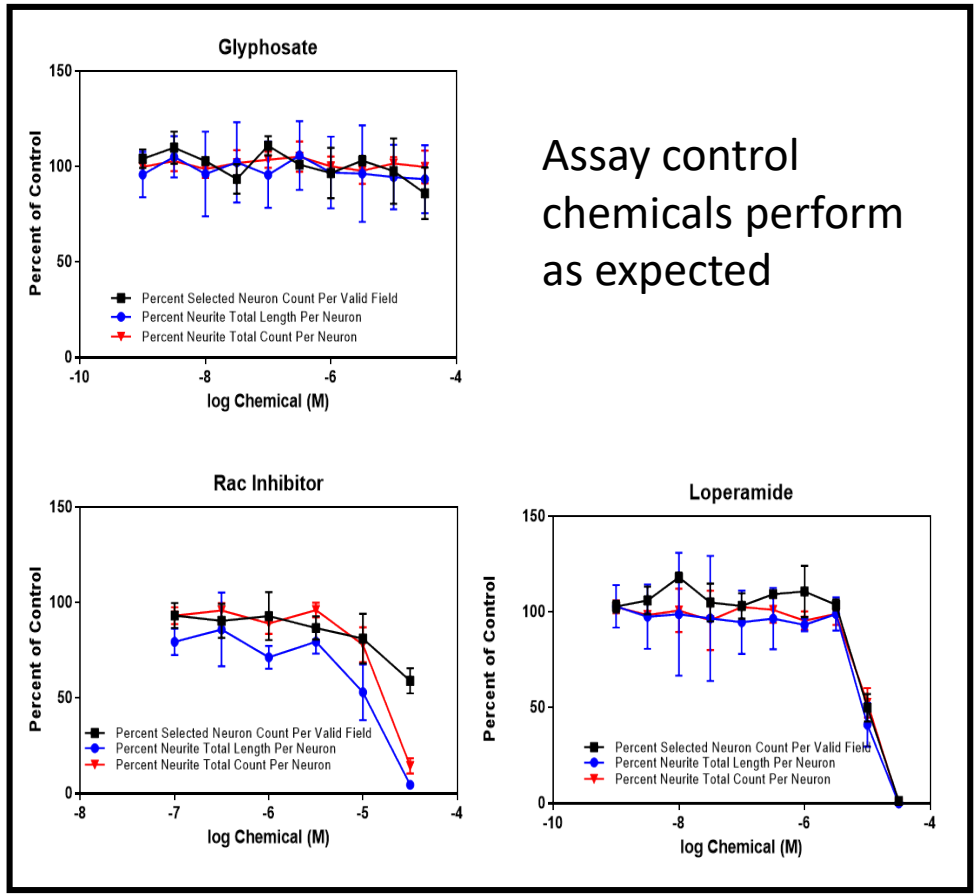
Compound X and analogs lack effects on Neurite Outgrowth









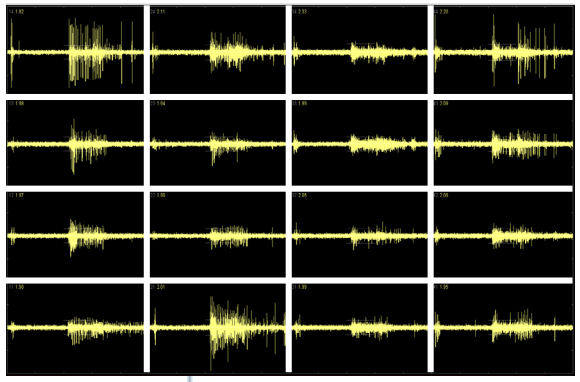


Assay control chemicals perform as expected

*EX000371 = Compound X

Conclusion: Compound X and analogs have no effects on neurite outgrowth

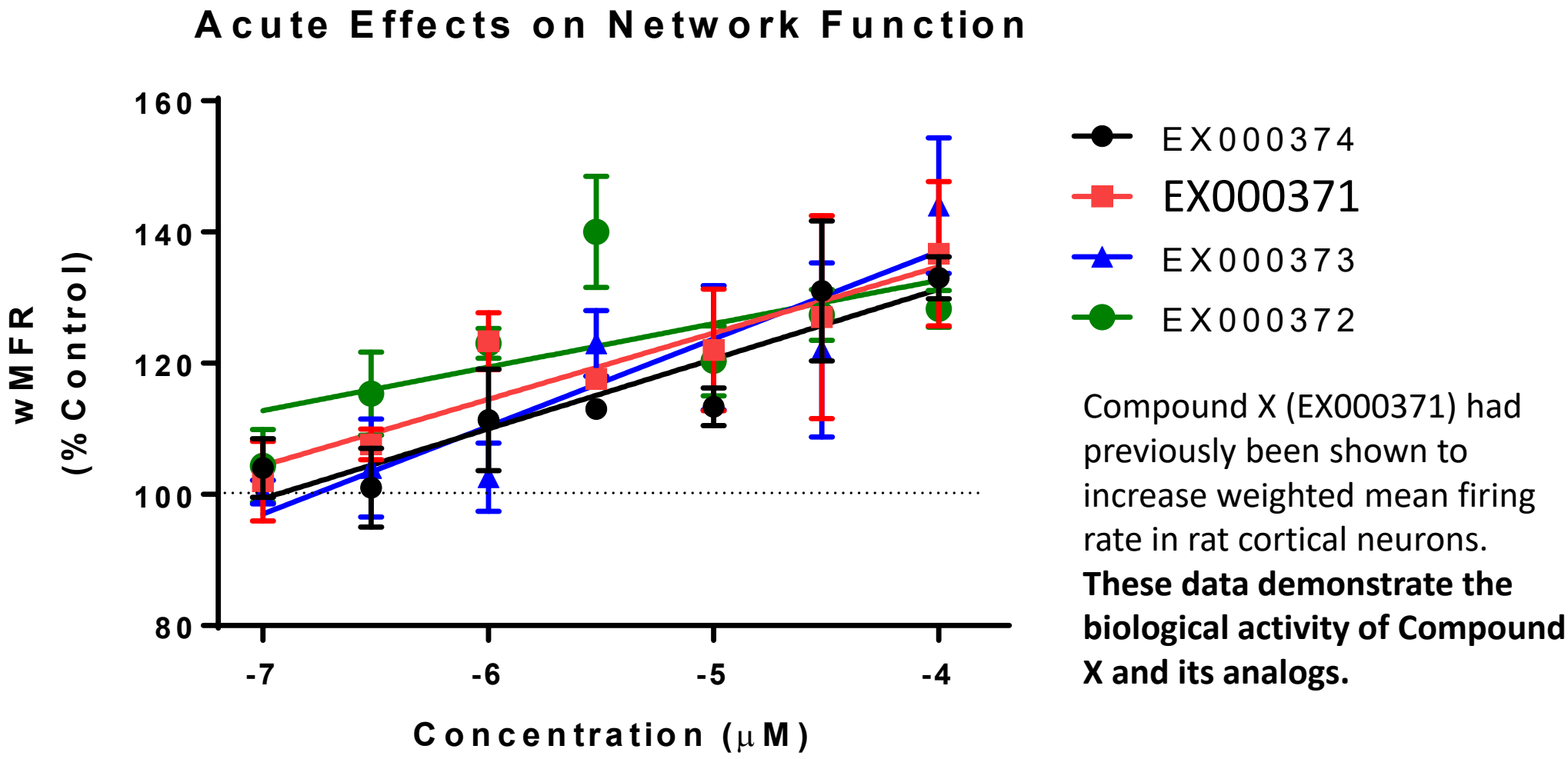
Compound X and analogs lack effects on Network Formation



Assay controls (Glyphosate; negative and Loperamide; positive) produce the expected results

Conclusion: Compound X (EX000371) and analogs have no effects on Network Formation

Compound X and analogs alter Network Function



Compound X (EX000371) had previously been shown to increase weighted mean firing rate in rat cortical neurons. **These data demonstrate the biological activity of Compound X and its analogs.**

IVIVE indicates appropriate concentration ranges were tested

From Guideline study, NOAEL of Compound X = **14 mg/kg/day**

Using HTTK and IVIVE

- 1 mg/kg/day = Css values of 0.66 and 2.21 µM in rats and humans, respectively
- 30 µM Compound X = AED of **45 mg/kg/day** (rats) and 13.5 mg/kg/day (humans)

Summary: At concentrations equivalent to or above the NOAEL from in vivo studies, Compound X and analogs did not alter Neurite Outgrowth or Network Formation, but did have acute effects on Network Function

These data, along with other (e.g. exposure) data, were used to support a decision by OPP to waive the requirement for a DNT Guideline study for compounds Y and Z

Example #3: Organophosphates and DNT

Organophosphate (OP) 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 Weight of Evidence (WOE) approach for OPs?

Example #3: Organophosphates and DNT

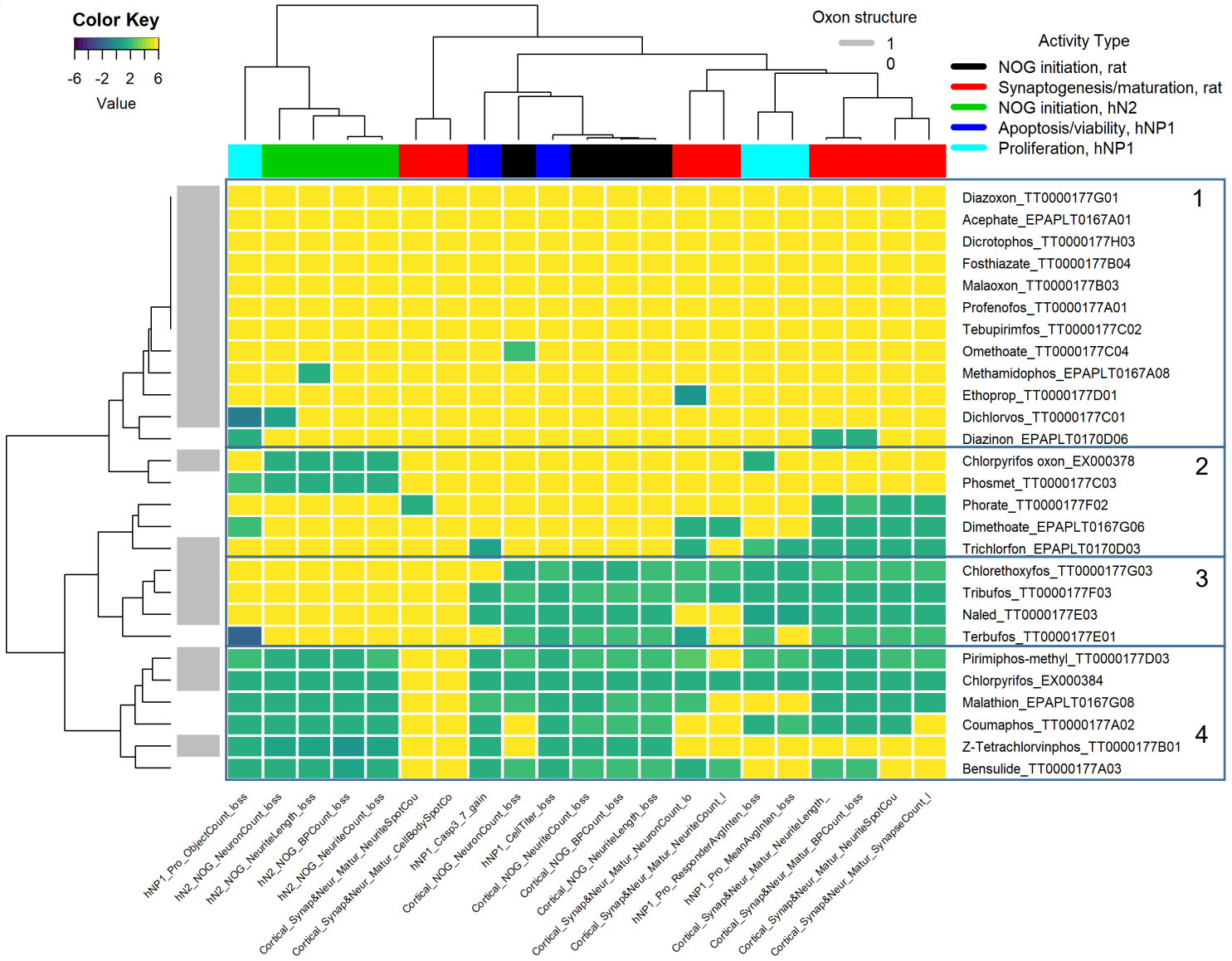
Study Design:

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:

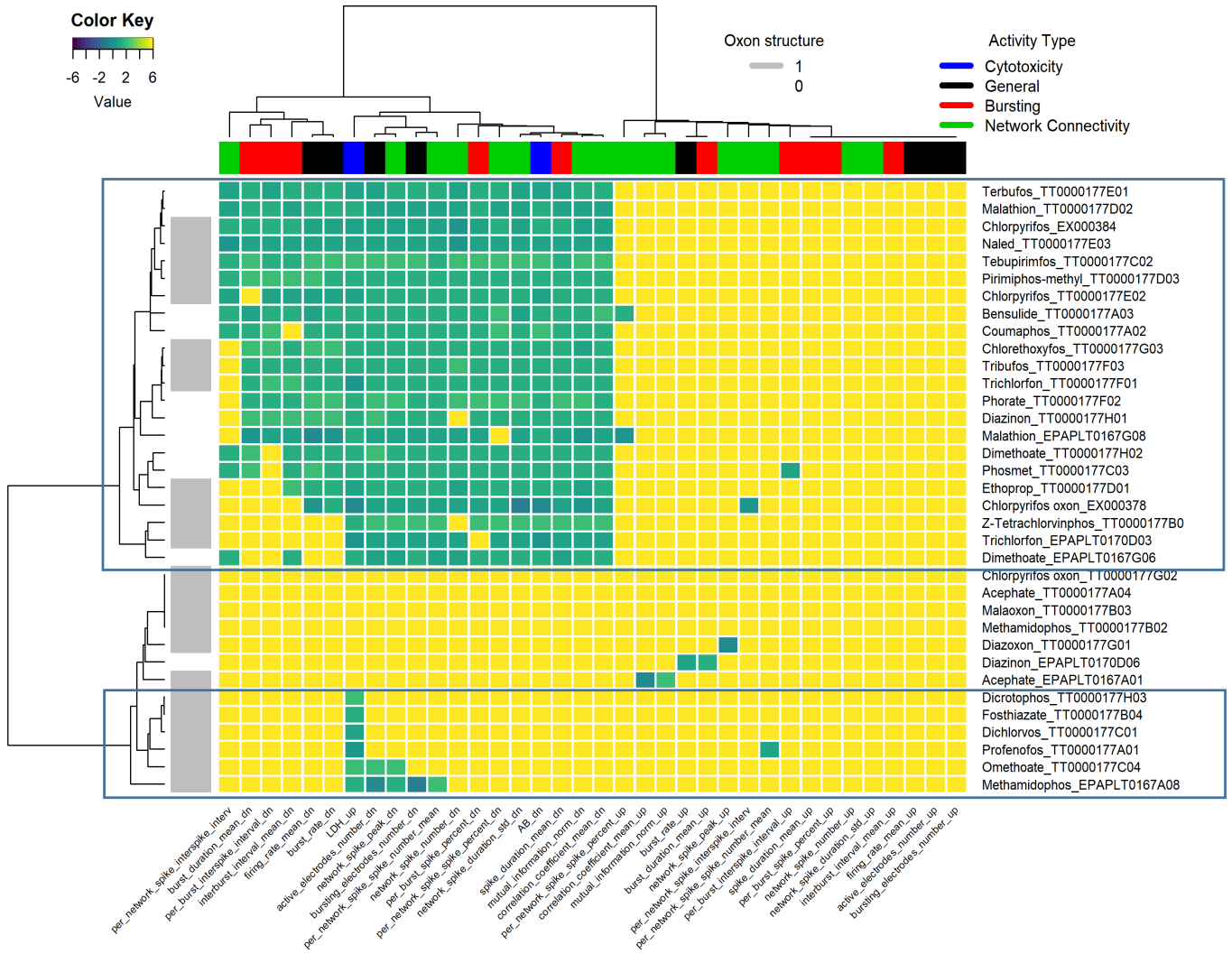
Proliferation	-	human neuroprogenitors (hNP1)	High-Content Imaging (HCI) assays
Apoptosis	-	human neuroprogenitors (hNP1)	
Neurite initiation	-	human neurons (hN2)	
Neurite initiation	-	rat primary neural culture	
Neurite maturation	-	rat primary neural culture	
Synaptogenesis	-	rat primary neural culture	
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: 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

HCI and MEA_NFA assays show consistent results

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

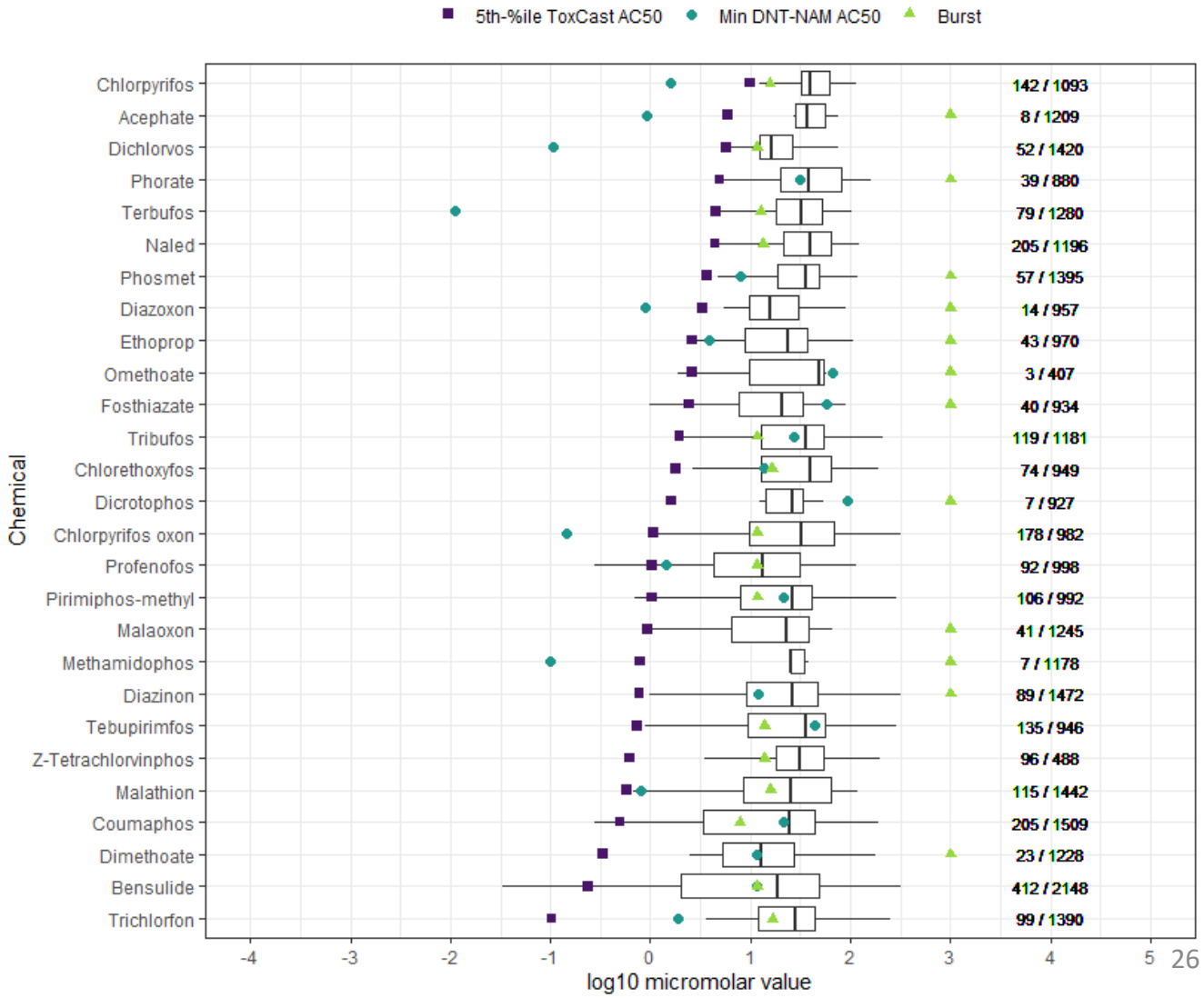
- *Equiv or Pos in MEA NFA and negative in HCI*: Acephate, diazoxon, dichlorvos, dicrotophos, fosthiazate, malaoxon, omethoate, profenofos
- *Positive in MEA NFA and negative in HCI*: Ethoprop
- *Positive in HCI and negative in MEA NFA:* OP chemical (methamidophos) was neg/equiv in the MEA NFA
- **If 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.

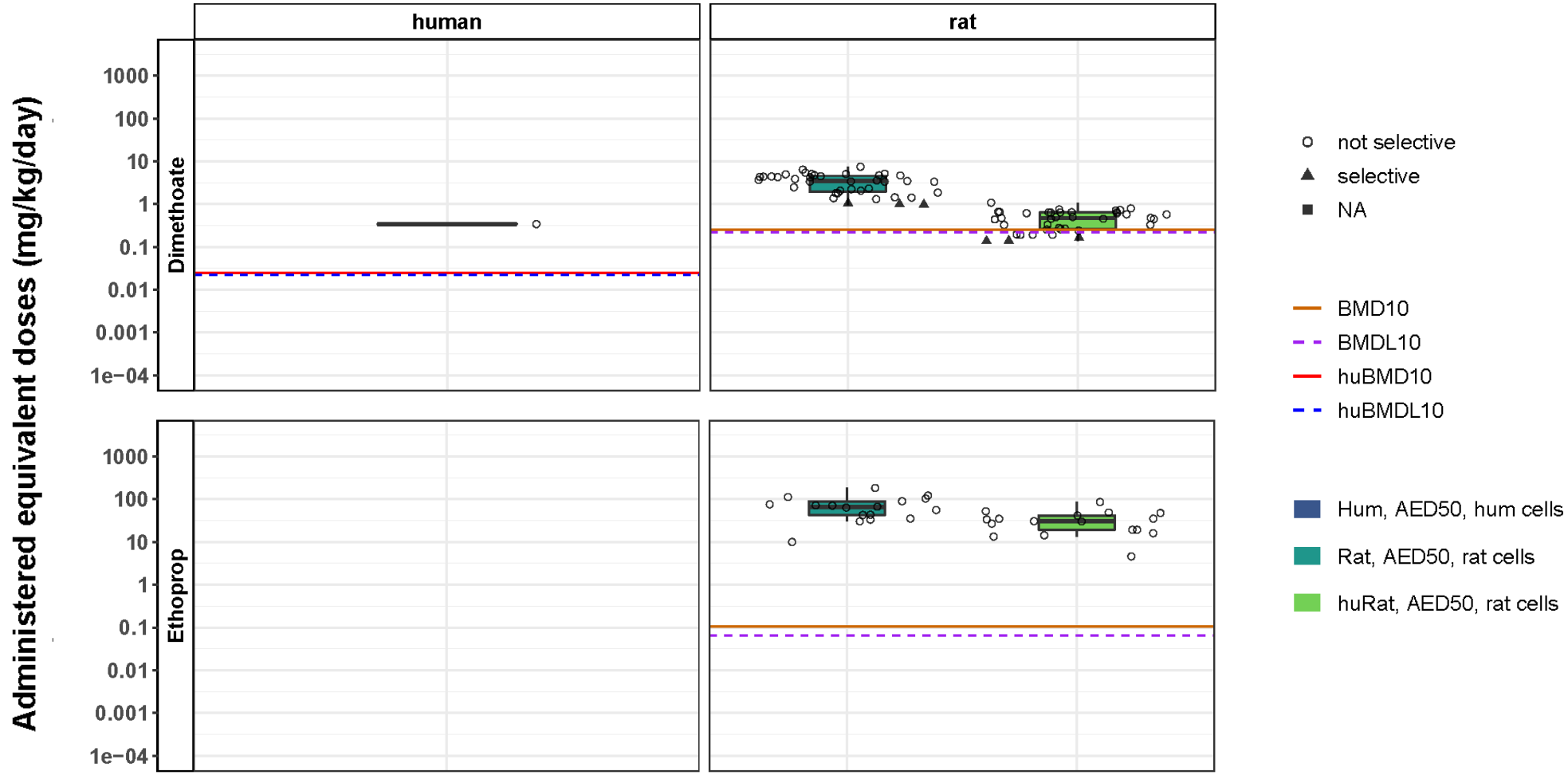
DNT-NAM battery may provide a more potent estimate of bioactivity for substances with minimum DNT-NAM AC50 < 5th percentile of filtered ToxCast AC50 values:

- 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.

Summary for OP effects in DNT NAMs

- Overall, the BMDs for AChE inhibition are lower than those for DNT NAMS
  - This **decreases uncertainty** that the AChE values are health protective

- DNT NAM AED50 values approached the AChE BMD values for some compounds (dichlorvos, dimethoate, malathion)

- In 2020, a Scientific Advisory Panel reviewed these data and determined that NAMs can be used as part of a weight of evidence approach for decision-making regarding DNT.

- Future Direction- these OPs will be tested in the other DNT NAMs in the battery in the next year.

Overall conclusion

The development of a DNT-NAM battery for assessing potential DNT hazard:

- Provides an opportunity to overcome some of the challenges with the *in vivo* DNT guideline
- Evaluates critical processes underlying neurodevelopment
- Incorporates human relevant information

DNT NAMs are being utilized at the EPA for a variety of regulatory decision-making processes

Thank you!
Questions?

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