Protecting the developing brain from harmful chemicals using in vitro approaches

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February 20, 2020

The Problem: Developmental Neurotoxicity (DNT) has been examined for too few chemicals

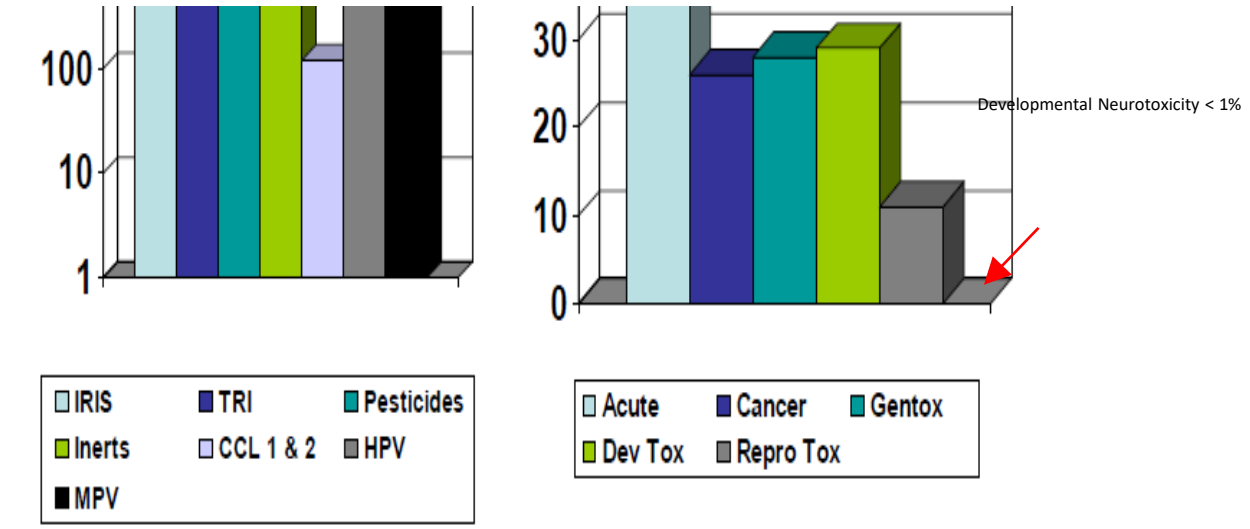
Current testing too slow

$1M

yrs

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

- Develop high throughput, in vitro assays,
- Screen and prioritize chemicals for developmental neurotoxicity hazard



In the absence of DNT hazard data, it is not possible to:

a) Evaluate the role of environmental chemicals in neurodevelopmental disease
b) Evaluate potential DNT risk for individual chemicals
c) Consider DNT as an adverse outcome in clean-up decisions at contaminated sites (e.g. Superfund sites).

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
Adminis

TO: Associat
General
Assistant
Inspecto
Chief Fi
Chief of
Associate Administrators
Regional Administrators

I am pleased today to establish the following commitments that will ensure our work in this area makes a real and significant difference. The EPA will reduce its requests for, and our funding of, mammal studies[1] by 30 percent by 2025 and eliminate all mammal study requests and funding by 2035. Any mammal studies requested or funded by the EPA after 2035 will require Administrator approval on a case-by-case basis. The EPA also will come as close as possible to

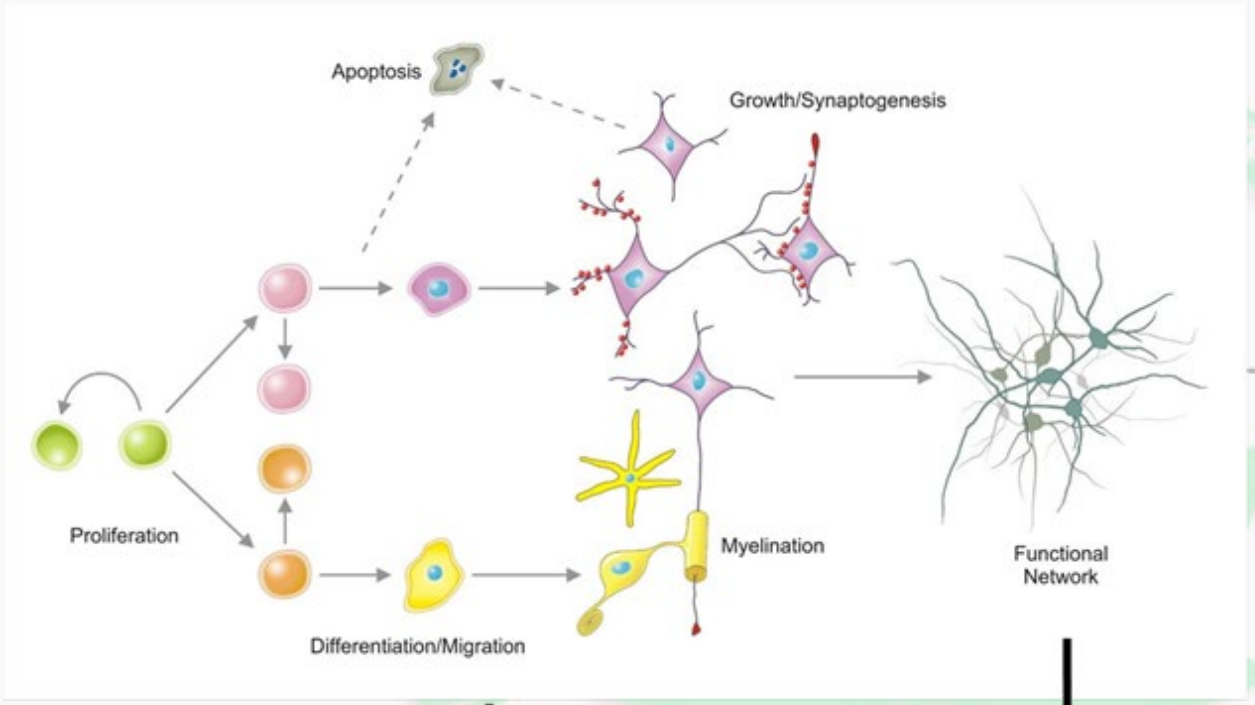
During my March 2019 all-hands address, I reiterated the U.S. Environmental Protection Agency's commitment to move away from animal testing. We are already making significant efforts to reduce, replace and refine our animal testing requirements under both statutory and strategic directives. For example, 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.

International Efforts to Develop Alternatives for DNT Guideline Studies

- European Food Safety Organization
  - Funding research to develop and evaluate a battery of in vitro DNT assays
- Danish EPA
  - Supporting evaluation of DNT alternatives
  - Combination of structural and functional endpoints
  - Qualification of primary hits by secondary testing (same assay; and hit confirmation testing using an alternative assay)
  - Integration of dosimetry to improve hit prediction from screening results
- National Toxicology Program (NTP)
  - Evaluating alternatives as a decision tool to best utilize limited resources for in vivo testing of nominated chemicals
  - Provided compounds for testing to a number of laboratories;
  - Built an interactive database (DNT DIVER) to house data and facilitate utilization of data for decision-making
- Organization for Economic Cooperation and Development (OECD)
  - DNT Expert Group
  - Guidance Document for Using NAMs for DNT IATAs

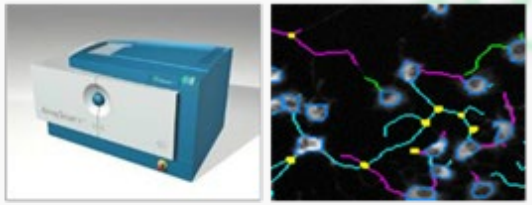
Phenotypic Screening for DNT Hazard

Quantify key neurodevelopmental events *in vitro*



Cognition Behavior

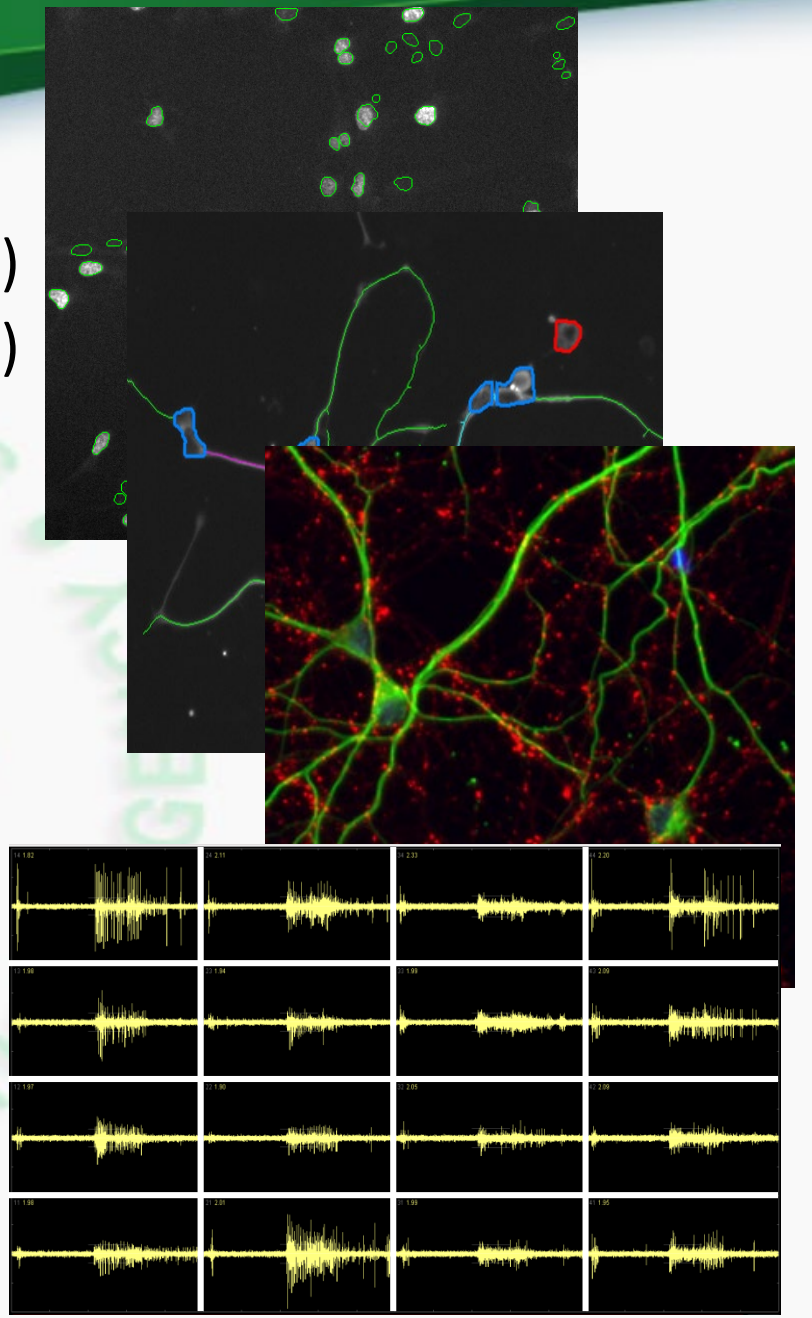
Key Neurodevelopmental Events (High Content Imaging)



Network Formation (Multi-Electrode Array)

EPA Assay Battery

Proliferation	-	human neuroprogenitors (hNP1)
Apoptosis	-	human neuroprogenitors (hNP1)
Neurite initiation	-	human neurons (hN2, iCell)
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



Each assay:

- Assay positive controls
- “DNT Reference” Compounds, known to cause DNT in vivo
- Concurrent measure of cell viability

Needs to Encourage Regulatory Use of Alternative Methods and for Guidance Document

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

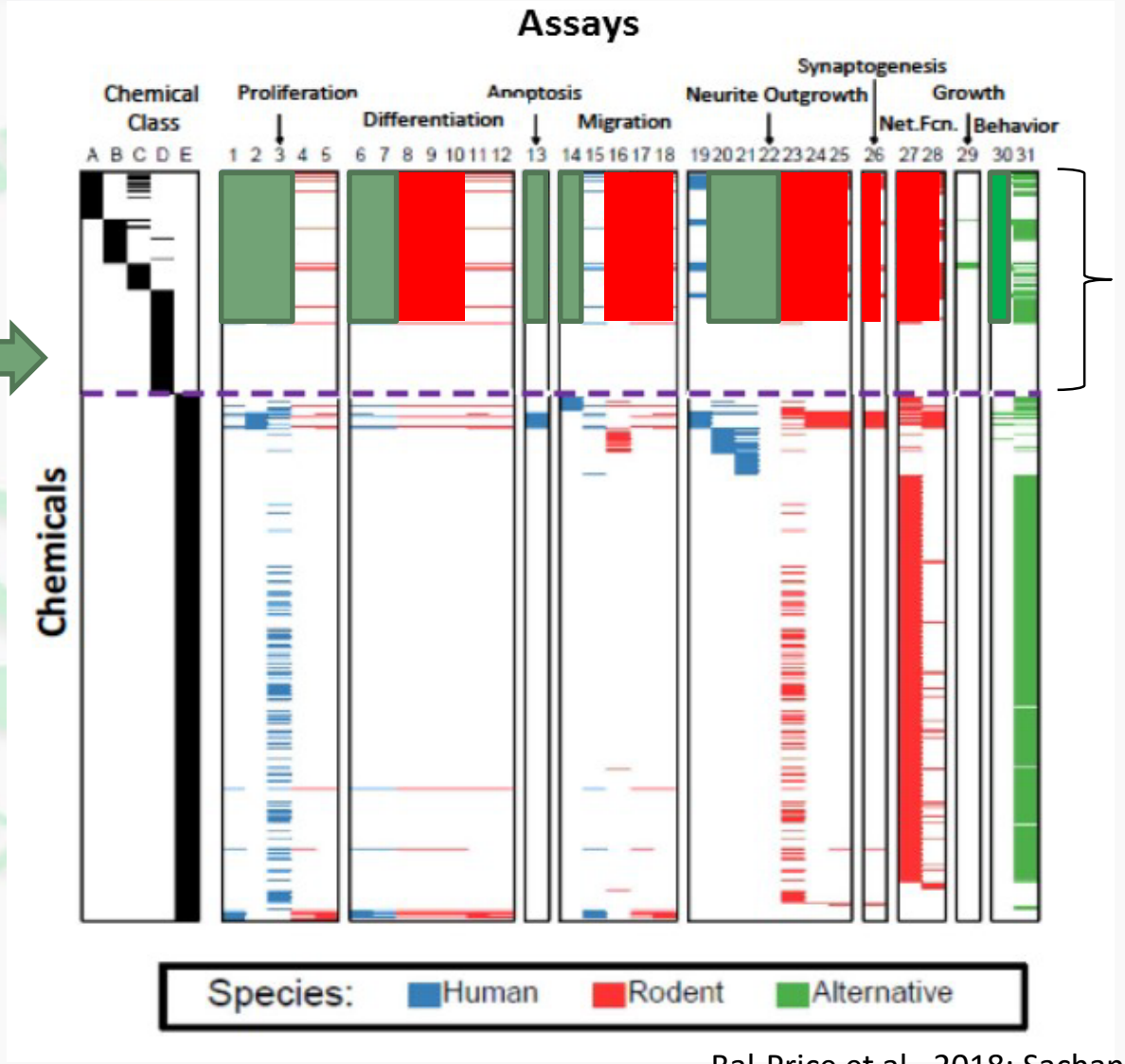
Regulatory decision-makers must have confidence in the assays and data in order to incorporate them into the decision-making process

The Need for More Data

Priority on compounds with in vivo DNT information

Assay-specific Compound Lists; Focused on in vivo DNT

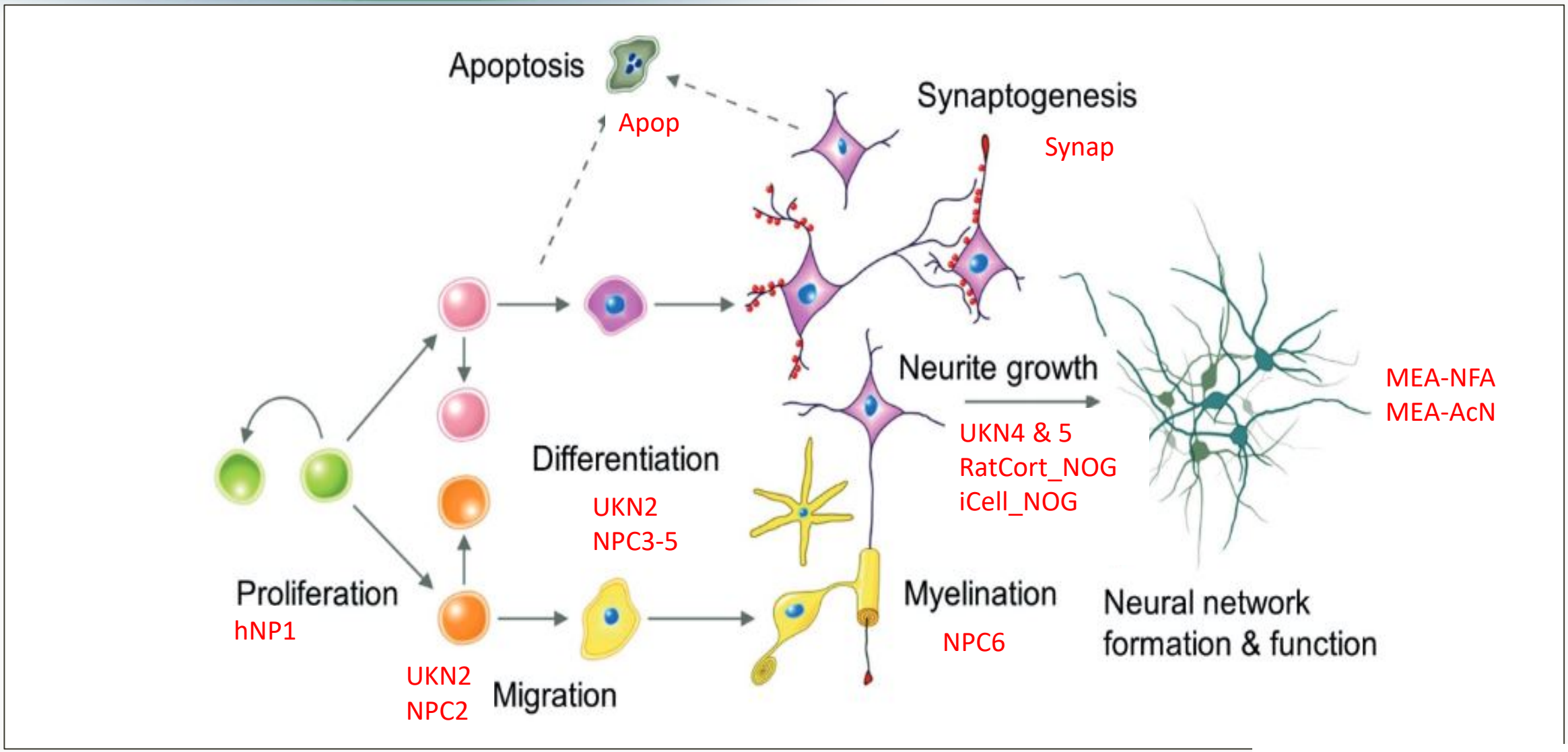
Assay 1
Assay 2
Assay3…



Assay Evaluation

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

In vitro Assays to Evaluate Chemical Effects on Neurodevelopmental Processes



Aschner et al., 2016

Confusion Matrix for In Vitro Assays

	Actual Positive	Actual Negative	Total
Predicted Positive	50	3	53
Predicted Negative	7	7	14
Total	57	10	67

- As additional data (from additional assays, zebrafish) becomes available, this will be updated.
- The preliminary indication is that the DNT in vitro Battery has a high sensitivity. The specificity of the battery may error on the side of being over-protective (increased false positive rate).

True Positive Rate (sensitivity) = True positives (50)/Known Positives (53) = **0.94**

True Negative Rate (specificity) = True negatives (7)*/Known Negatives (14) = **0.5**

Three of these have known in vivo neuroactivity: 7/11 =0.64

If **Selective** Effects are Considered:

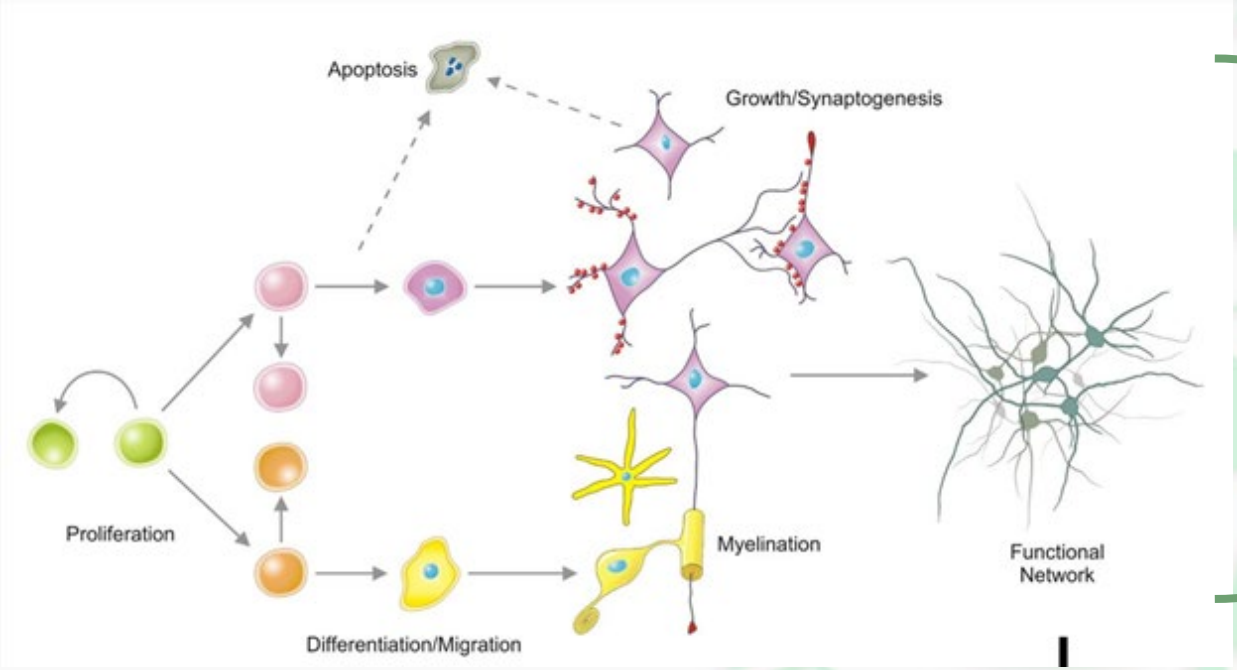
Sensitivity = 40/53 = 0.75

Specificity = 9/11 = 0.82

How can data from these assays be used?

How can I get DNT information on the thousands of compounds that have not been tested?

Using these assays to prioritize hazard testing for thousands of compounds



1000 Compounds + Screening Battery

ACTIVE?

ACTIVE → Prioritize for Additional Screening

Guidance will need to be developed on how to combine information across all assays in the battery

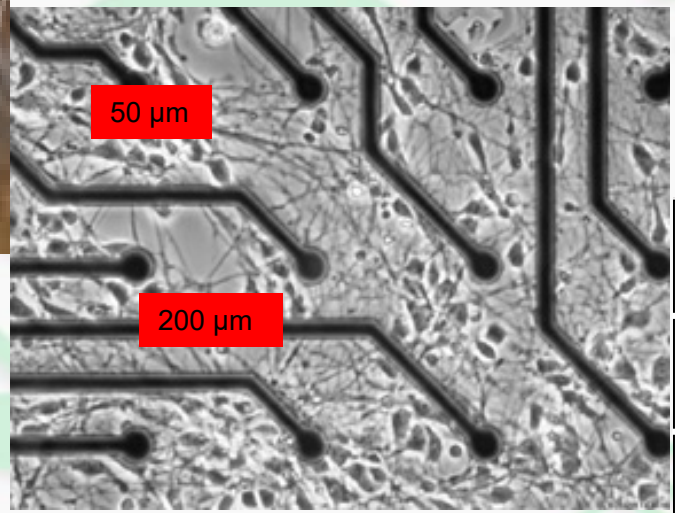
NOT ACTIVE → Low Priority

Example: TSCA chemicals

How might data from in vitro assays be used for DNT testing?

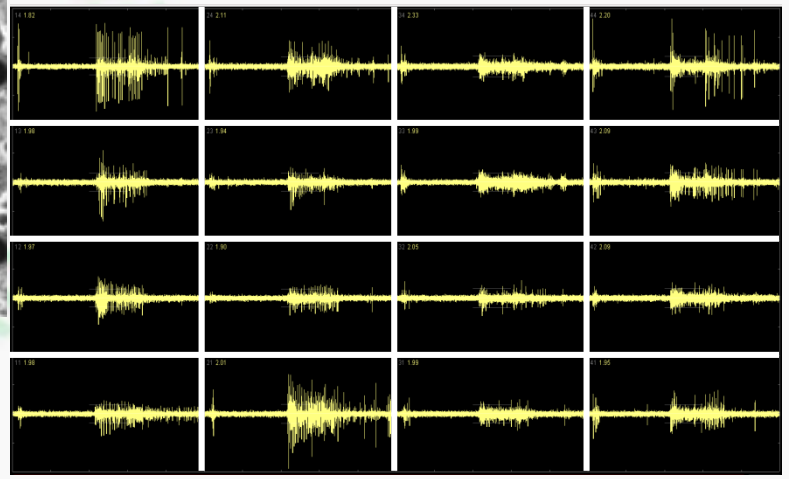
How do I prioritize the positives?

Functional measurement of network activity in vitro using Microelectrode Array (MEA) Recording



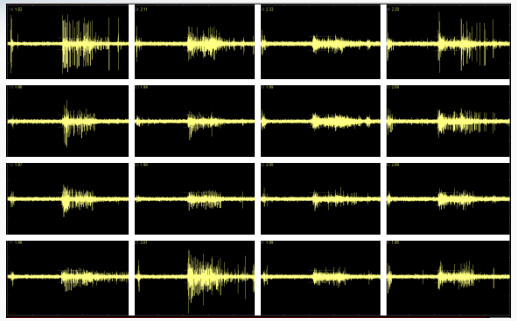
“Brain-on-a-Chip”: Complex 2D model

- 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.

Network Formation Assay (NFA)



Days in Vitro- toxicant present throughout

0 — 2 — 5 — 7 — 9 — 12

Record / Change Media (2)

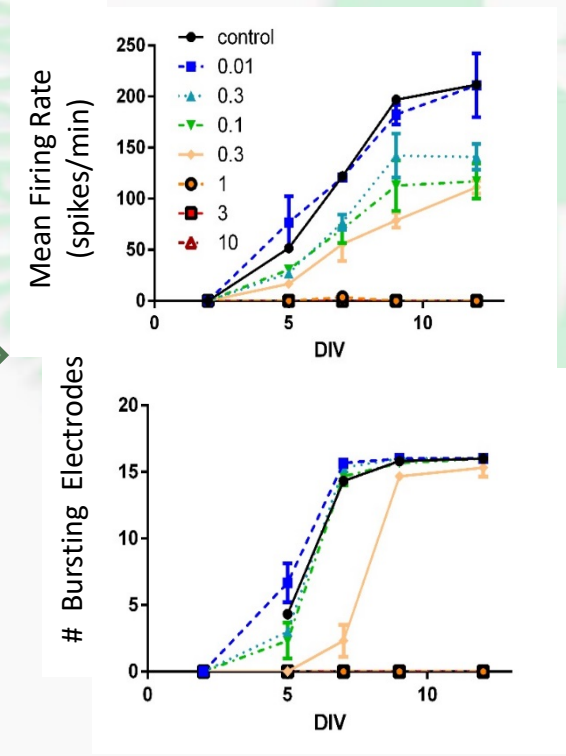
Record (5)

Record / Change Media (7)

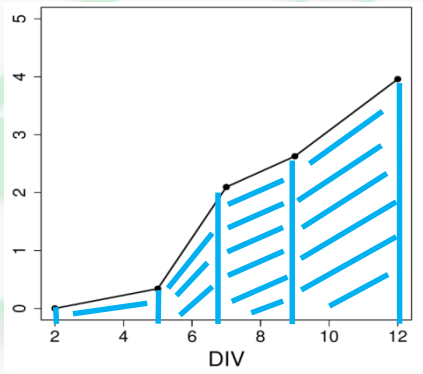
Record Viability (12)

General Activity	Mean Firing Rate (MFR)
	Burst Rate (BR)
	Number of Active Electrodes (#AE)
	Number of Actively Bursting Electrodes (#ABE)
Bursting Activity	Interspike Interval (ISI) within a burst
	Percentage of Spikes in Burst (%SiB)
	Mean Burst Duration (BD)
	Mean interburst interval (IBI)
Network Connectivity	Number of Network Spikes (#NS)
	Network Spike Peak (NSP)
	Network Spike Duration (NSD)
	SD of Network Spike Duration (NSDsd)
	ISI in Network Spike (NS-ISI)
	Mean number of Spikes in Network Spikes (#SiNS)
	% Spikes in Network Spike (%SiNS)
	Mean Correlation (r)
	Normalized Mutual Information

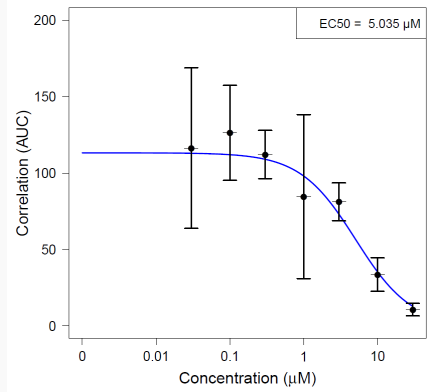
Methylmercury



Determine Area Under the Curve for each dose (8) and parameter (17)



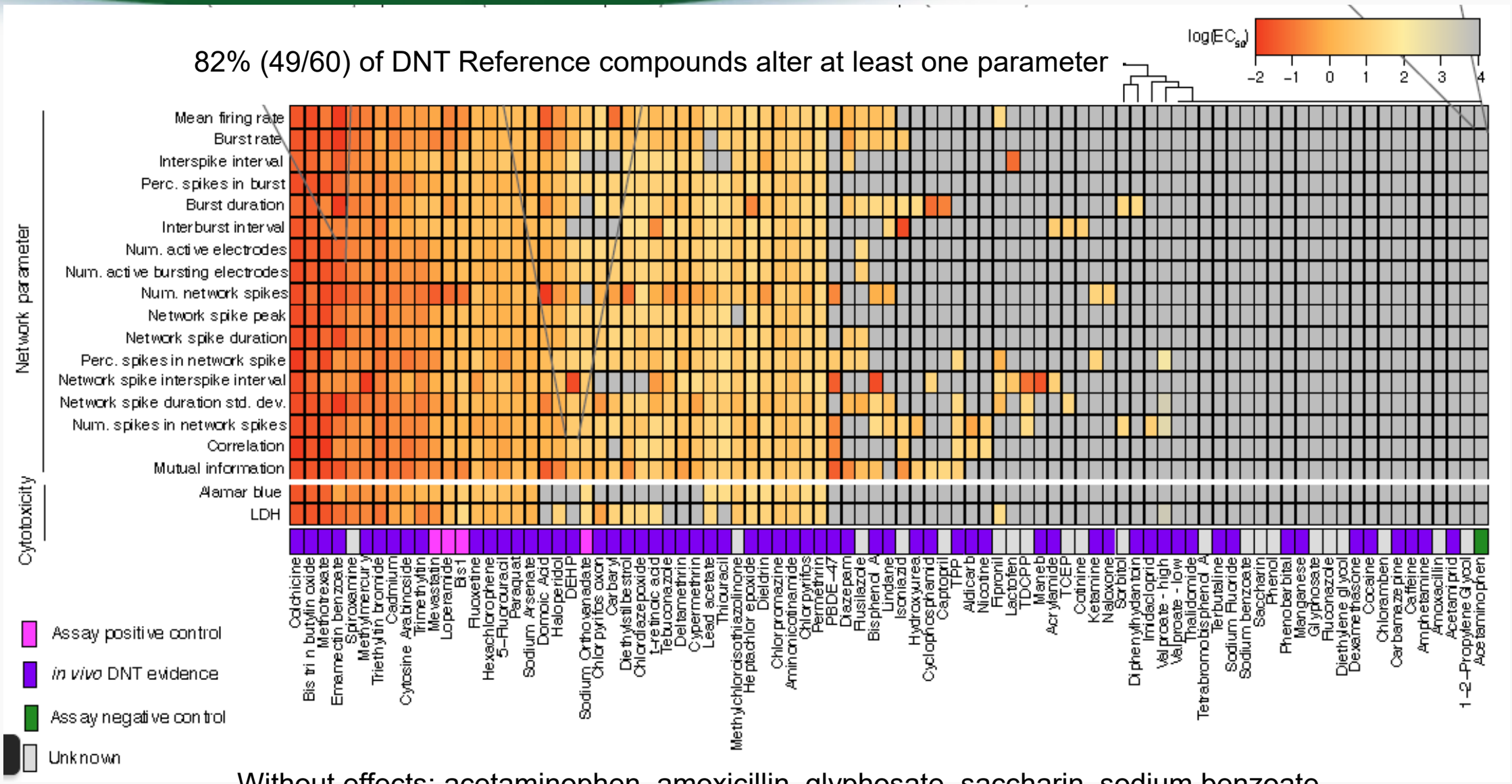
Determine concentration-response



Based on Frank et al., Toxicol Sci, 2017.

The Assay can separate developmentally neurotoxic from non-neurotoxic compounds

82% (49/60) of DNT Reference compounds alter at least one parameter



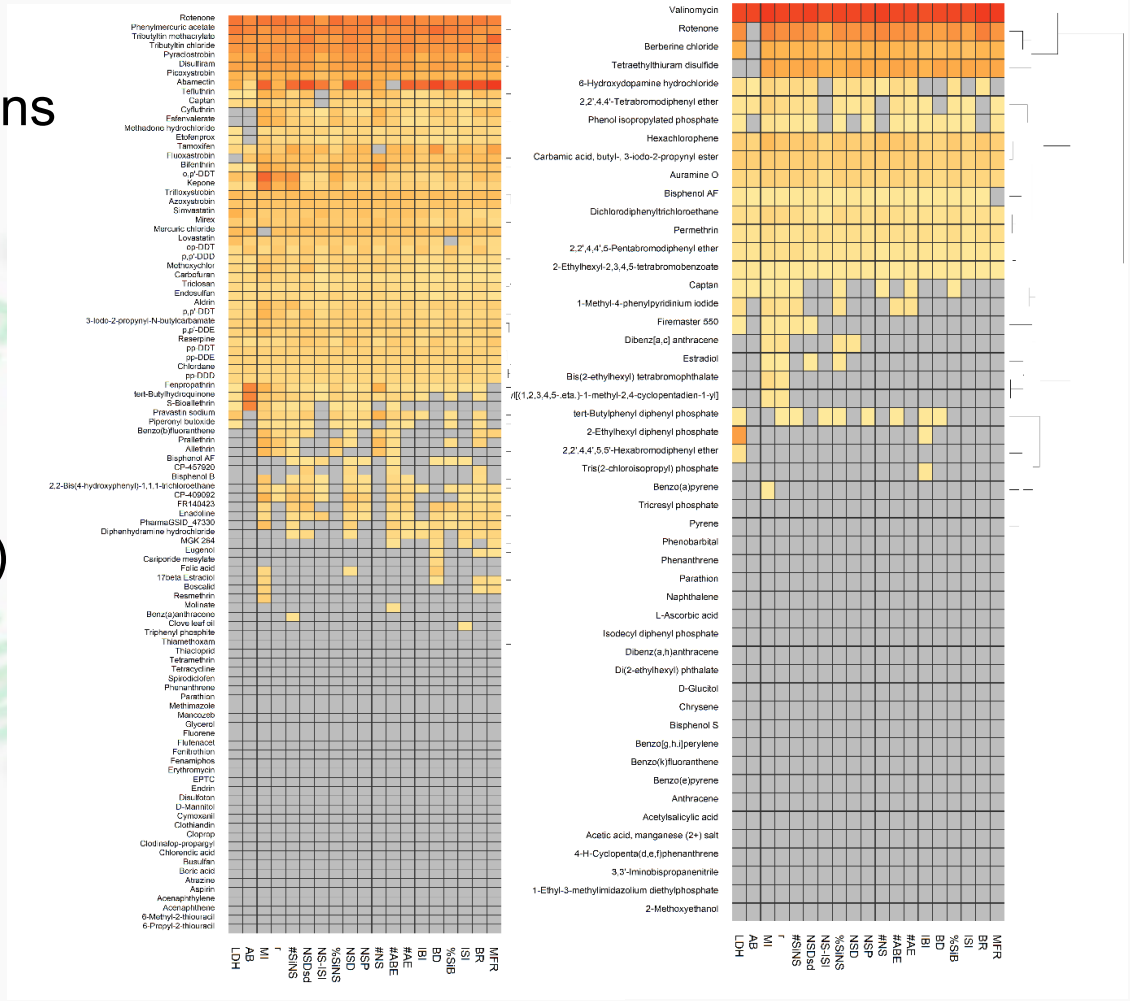
Without effects: acetaminophen, amoxicillin, glyphosate, saccharin, sodium benzoate.

Additional Screening in MEAs

Screened 154 unique compounds, including:

- 17 Polyaromatic Hydrocarbons
- 22 Pharmaceuticals
- 13 Flame Retardants
- 12 Pyrethroids
- 11 Organochlorines
- 32 Organophosphates
- 3 Neonicotinoids
- 11 Fungicides (5 strobilurins)
- 4 Herbicides

- 8 negative controls



Quick Summary:

- Good sensitivity and specificity
- Good replicability
- Possible to screen hundreds of compounds

But…. No information on underlying mechanisms

Data for 137 of these compounds: Shafer et al., ToxSci.in press Feb 13, 2019

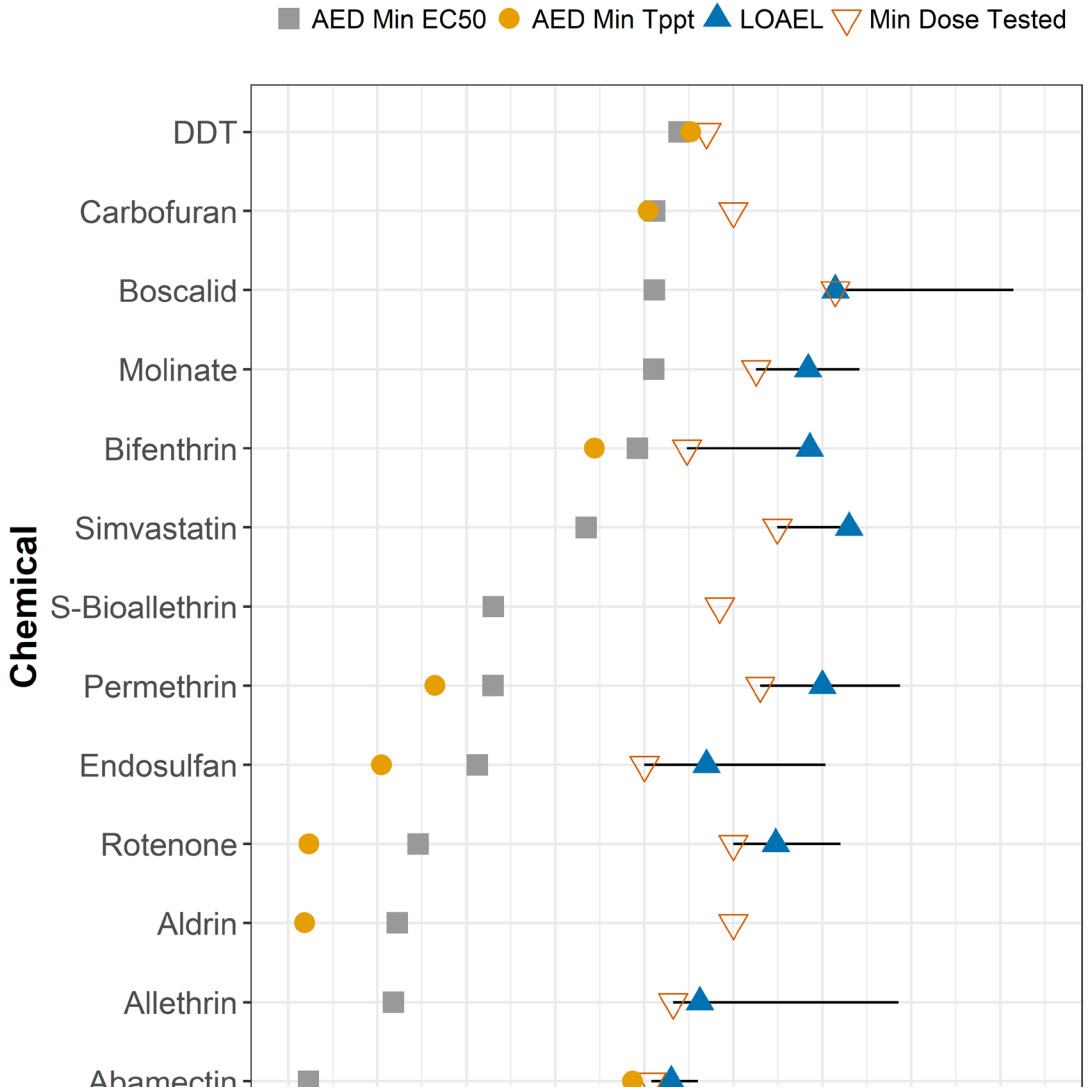
How might data from in vitro assays be used for DNT testing?

How can compounds that might target the nervous system specifically be identified?

How do the in vitro effects compare with in vivo?

A

Extrapolate other ToxCast Assays*



B

These data demonstrate:

1) **Assays fill a biological gap in ToxCast assays**
2) **Data could be used to identify compounds of concern for neurotoxicity/DNT**

These data demonstrate:

1) **Assays provide estimates of activity that are relevant to in vivo DNT effects.**
2) **Prediction model needs to include information regarding exposure.**

Shafer et al., Toxicol Sci., 2019.

Summary and Conclusions

- Testing chemicals for DNT hazard using in vitro approaches is being encouraged
  - Addresses need for data on thousands of compounds
  - Faster and less expensive than conventional studies

- In vitro approaches to DNT testing can provide useful information
  - Biological activity of compounds
    - Active/not-active
    - Potency and ranking of actives
  - Comparison of biological activity towards nervous system vs other toxicities
  - In Vitro to In Vivo Extrapolation (IVIVE)

The Team

EPA

- Theresa Freudenrich
- Kathleen Wallace
- Jasmine Brown
- Chris Frank
- Diana Hall
- Chris Grant
- Stephanie Padilla
- Bill Mundy (retired)
- Kevin Crofton (retired)
- Katie Paul-Friedman
- Richard Judson

NIEHS/NTP

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- Fred Parham

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- Marcel Leist
- Johanna Nyffeler

Düsseldorf

- Ellen Fritsche

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