Evaluation of *in vitro* New Approach Methodologies for Developmental Neurotoxicity

Kelly Carstens, PhD

U.S. Environmental Protection Agency
Research Triangle Park, NC

Email: carstens.kelly@epa.gov

ORCiD: 0000-0002-1746-5379

Office of Research and Development
Center for Computational Toxicology and Exposure
Biomolecular and Computational Toxicology Division
Computational Toxicology and Bioinformatics Branch

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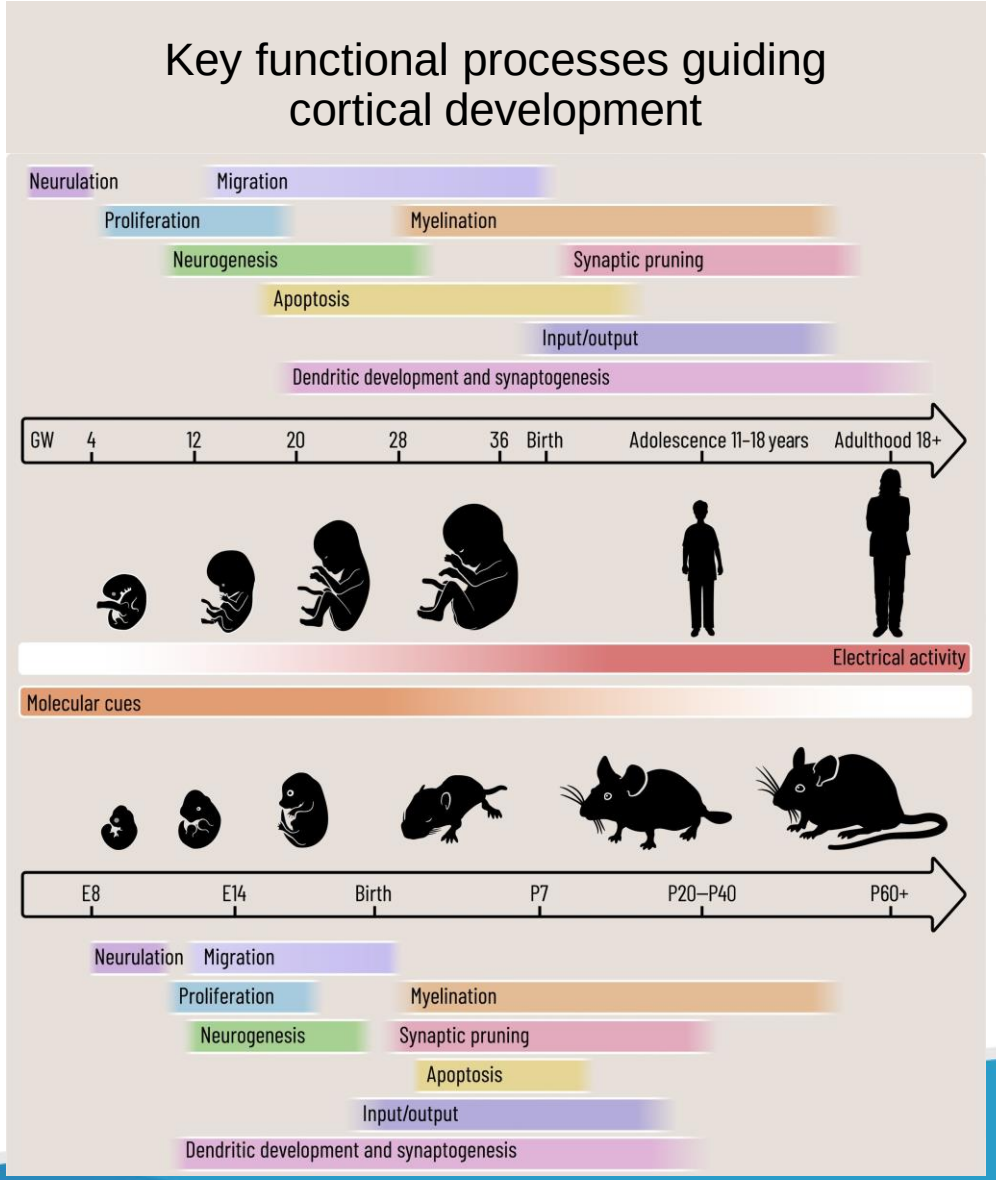
Conflict of Interest Statement

The views expressed in this presentation are those of the authors and do not necessarily reflect the views or policies of the U.S. EPA.

Background on developmental neurotoxicity (DNT) new approach methods (NAMs)

- Neurodevelopmental disability is the most prevalent chronic medical condition encountered in pediatrics (Zablotsky et al. 2019).
- Both **genetic and environmental risk factors** have been identified as underlying causes driving this prevalence.

- DNT NAMs battery: multi-dimensional DNT screening assays that cover complex neurobiological space: temporal, different 'key events' in neurodevelopment, cell-types, and species.
- Challenges in evaluating DNT NAMs:
  - No single *in vitro* screening assay can recapitulate all critical cellular events of neurodevelopment.
  - Some compounds may disrupt specific cellular events at different stages of development.
  - Some neural cell-types may be differentially sensitive to perturbation.



Chini and Hanganu-Opatz. 2021. Trends in Neuro.

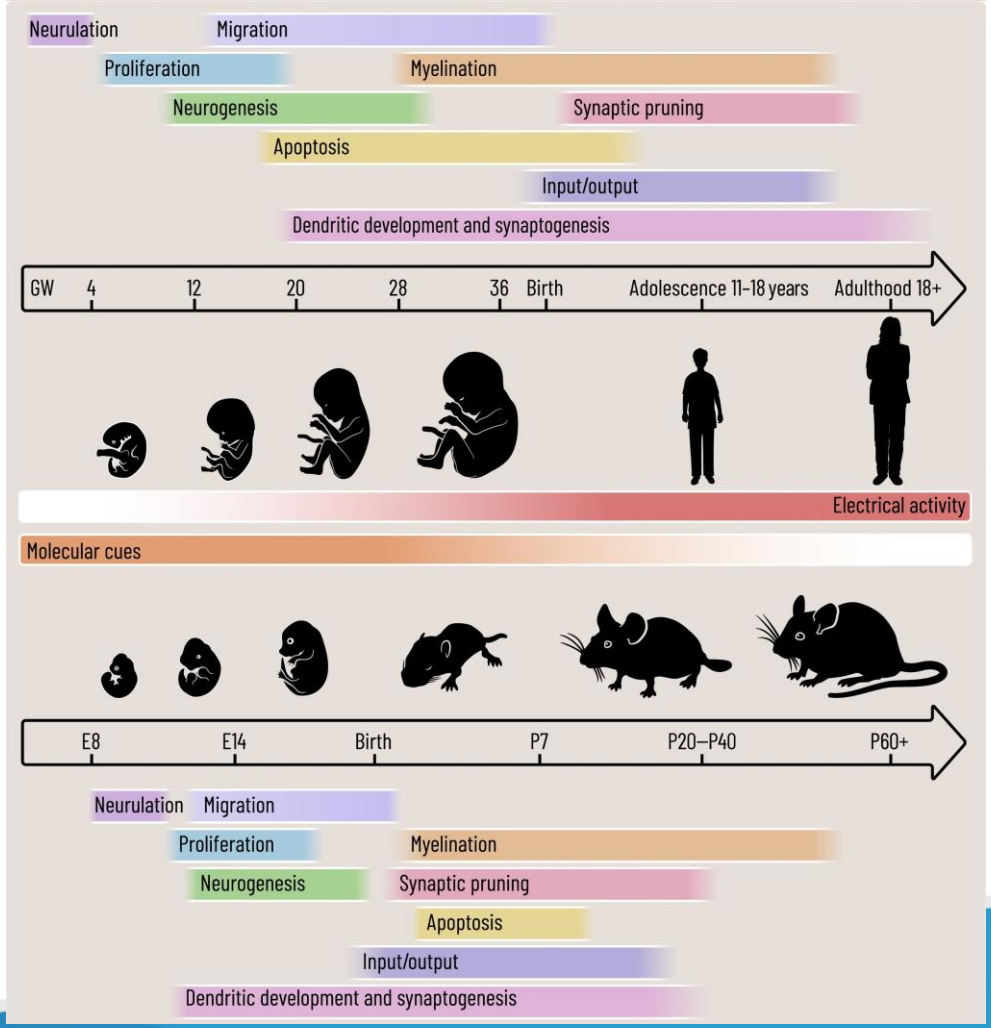
Overview

1) How does a broad screening battery collectively inform DNT-relevant bioactivity?

2) Can we build a model to classify compounds that demonstrate *in vivo* DNT bioactivity?

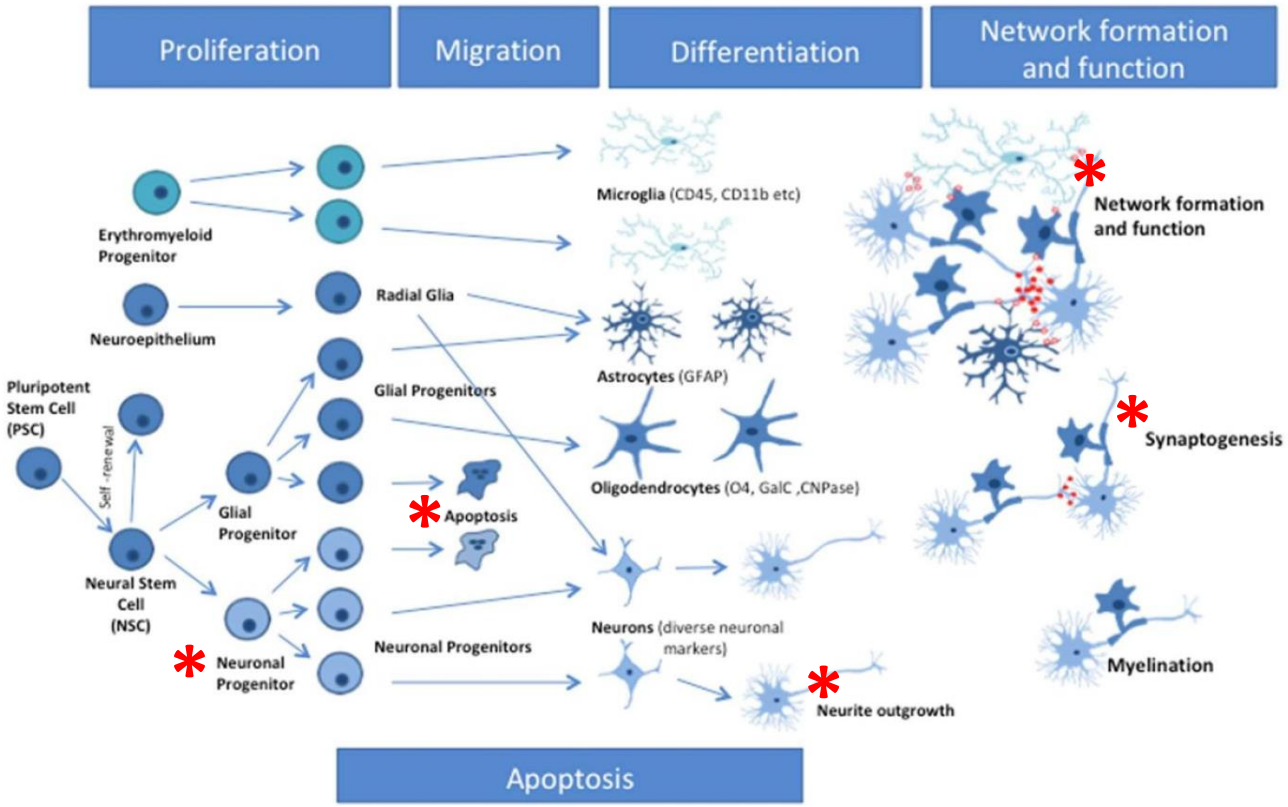
3) Can we identify biological gaps in the current EPA DNT NAM battery and/or broader ToxCast/ Tox21 database?

Key functional processes guiding cortical development



Chini and Hanganu-Opatz. 2021. Trends in Neuro.

Neurodevelopmental processes in the EPA DNT NAM battery



Bal-price et al. 2018

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

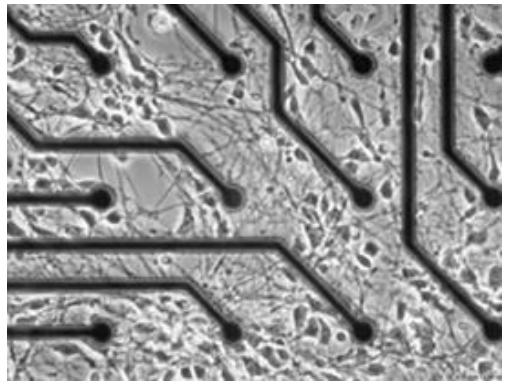
Process	Assays	References
Proliferation	hNP1	Harrill *et al.* (2018)
	NPC1	Baumann *et al.* (2016) and Barenys *et al.* (2017)
	UKN1	Balmer *et al.* (2012)
Apoptosis	hNP1	Harrill *et al.* (2018)
Migration	NPC2	Baumann *et al.* (2016) and Barenys *et al.* (2017)
	UKN2	Nyffeler *et al.* (2017)
Neuron differentiation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

Sachana, M., et.al. 2019, Toxicological Sciences

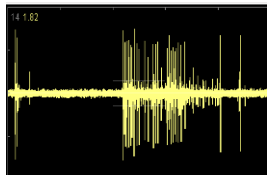
Experimental models in the EPA DNT NAM battery

Microelectrode Array (MEA) Network Formation Assay (NFA)

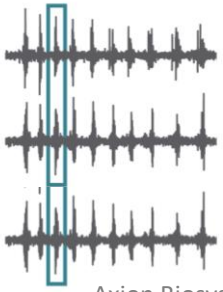
48-well culture plate
16 electrodes per well



Bursting



Connectivity



Axion Biosystems

Cell culture	Activity type	# endpoints
Primary rat cortical neurons (DIV 5, 7, 9, 12)	General activity	4
	Network connectivity	8
	Bursting	5
	Cytotoxicity	2

← 92 chemicals →

High Content Imaging

96-well culture plate
Immunohistochemistry

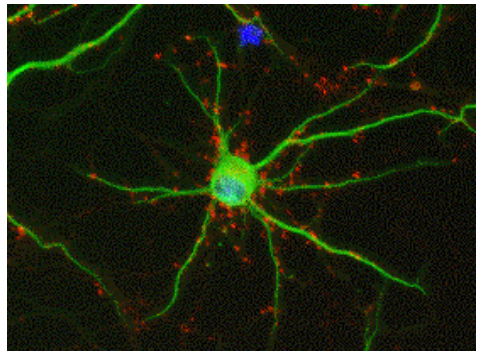
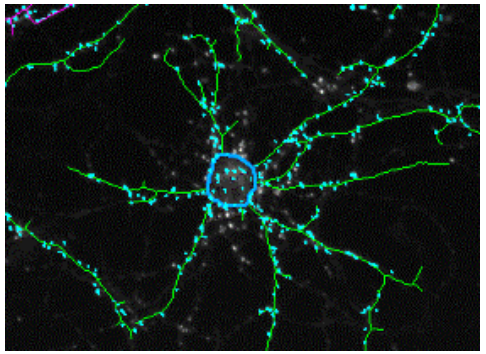


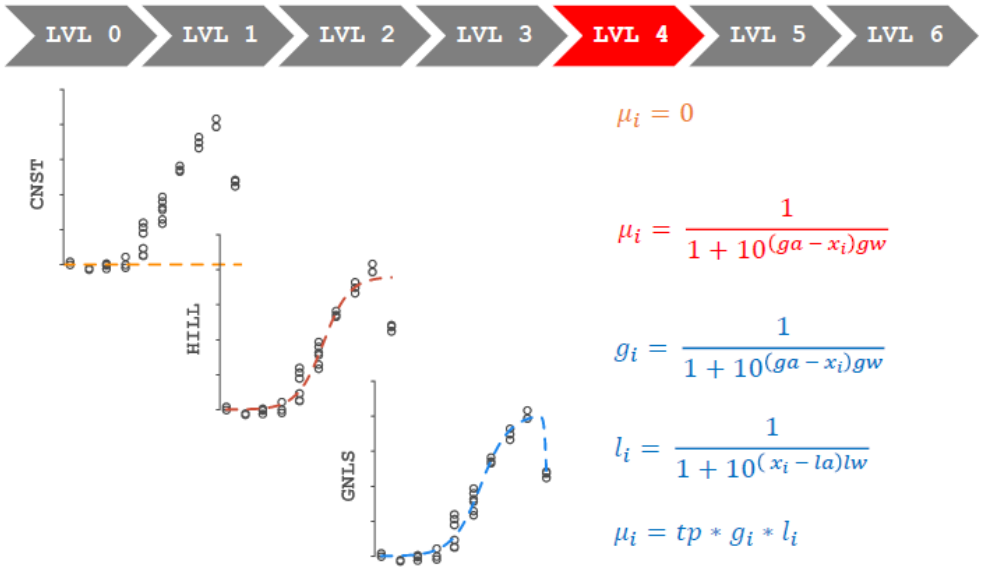
Image Analysis



Cell culture	Assays/ Key events	# endpoints
Primary rat cortical neurons	Neurite Outgrowth (NOG)	4
	Synaptogenesis and Neurite maturation	8
Human hN2 neural cells	NOG	4
Human hNP1 neuroprogenitors	Proliferation	3
	Apoptosis	2

Defining bioactivity using the ToxCast pipeline

Model fitting (constant, hill, gain-loss)



$$\mu_i = 0$$

$$\mu_i = \frac{1}{1 + 10^{(ga - x_i)gw}}$$

$$g_i = \frac{1}{1 + 10^{(ga - x_i)gw}}$$

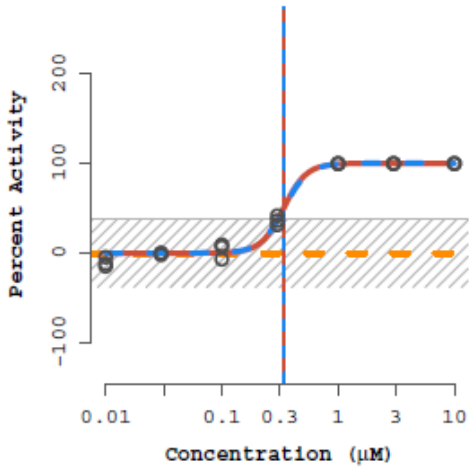
$$l_i = \frac{1}{1 + 10^{(x_i - la)lw}}$$

$$\mu_i = tp * g_i * l_i$$

https://cran.r-project.org/web/packages/tcpl/vignettes/Data_processing.html#level-4

Select winning model and hit-calling

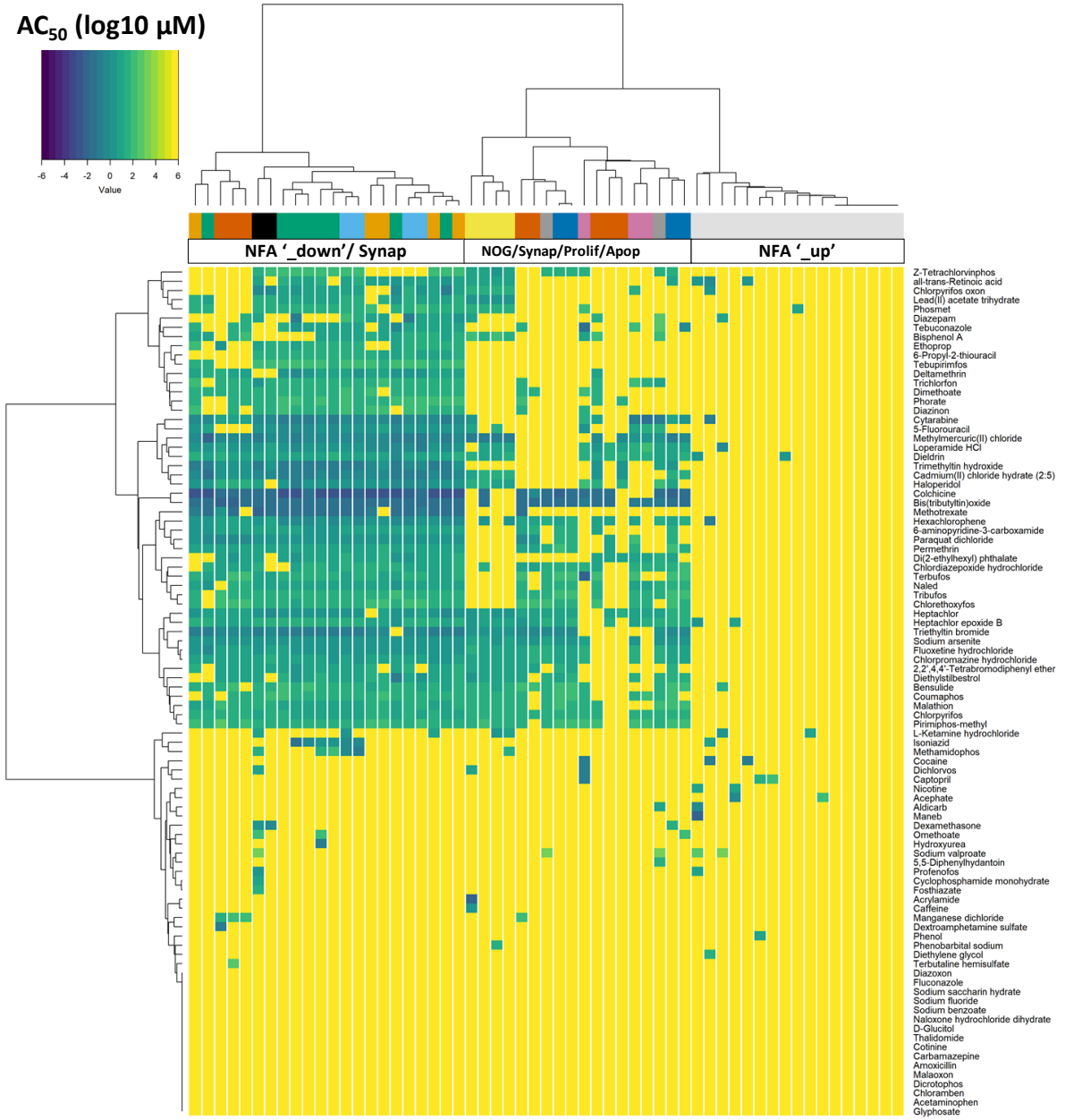
Number of bursting electrodes (down)




```
ASSAY:    AEID2500 (CCTE_Shafer_MEA_dev_bursting_electro

NAME:     Methylmercuric(II) chloride
CHID:     20813    CASRN: 115-09-3
SPID(S): EX000393
M4ID:     42619839

HILL MODEL (in red):
       tp        ga        gw
val:  100      -0.469    4.43
sd:   1.28     0.0287    2.18

GAIN-LOSS MODEL (in blue):
       tp        ga        gw        la        lw
val:  101      -0.465    4.21      1.22      10.2
sd:   NaN      NaN       NaN       NaN       NaN

       CNST        HILL        GNLS
AIC:  240.39      135.21      139.17
PROB: 0           0.88        0.12
RMSE: 67.13       5.4         5.39

MAX_MEAN: 100       MAX_MED: 100       BMAD: 12.8

COFF: 30.3    HIT-CALL: 1    FITC: 41    ACTP: 1

FLAGS:
```

ToxCast pipeline (tcpl) R package (version 2.0.3 publicly available) (Filer et al. 2017)

How does a broad screening battery collectively inform DNT-relevant bioactivity?



Activity Type

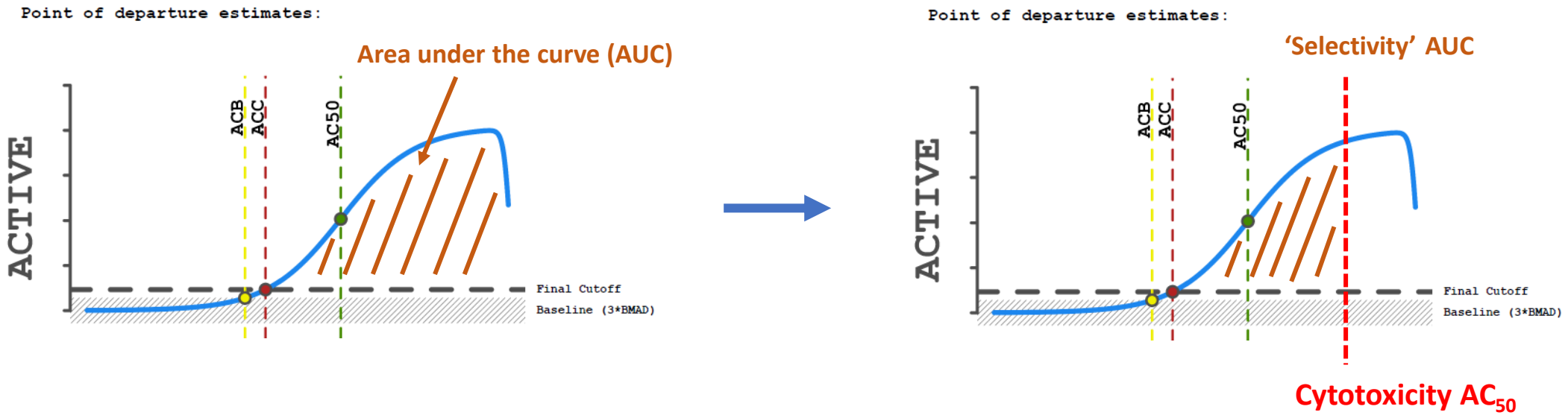
- NOG initiation, rat
- Synaptogenesis/maturation, rat
- NOG initiation, hN2
- Apoptosis, hNP1
- Proliferation, hNP1
- Cytotoxicity MEA
- General
- MEA NFA '_up'
- Bursting
- Network Connectivity

NFA: Network formation assay
Synap: Synaptogenesis
NOG: Neurite outgrowth
Prolif: Proliferation
Apop: Apoptosis

Carstens et al. 2022

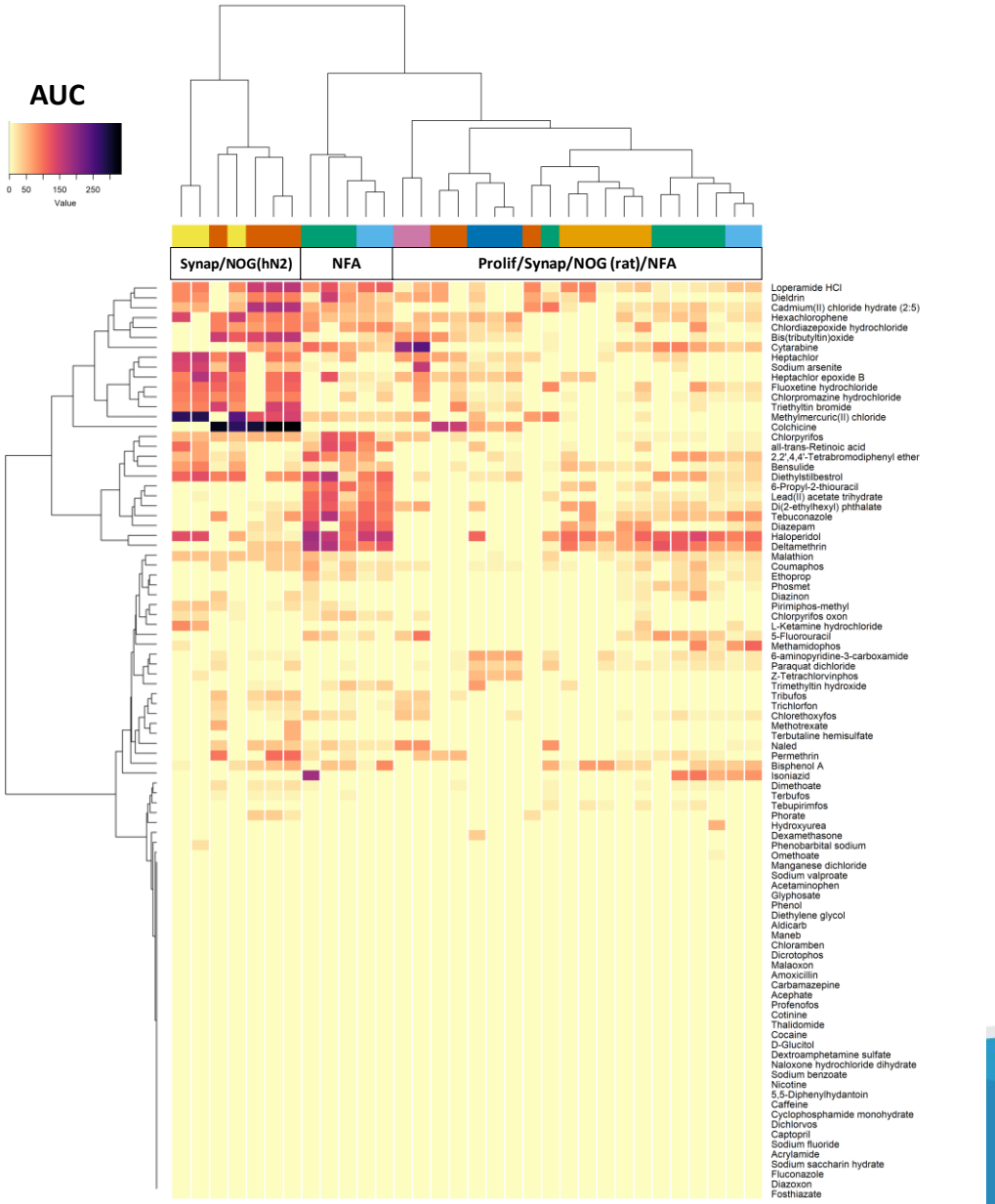
Selectivity: activity at concentrations lower than cytotoxicity

Calculating a ***selectivity*** metric:



https://cran.r-project.org/web/packages/tcpl/vignettes/Data_processing.html#level-4

Evaluating ***selectivity*** is informative for identifying patterns of activity.



Activity Type
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NFA: Network formation assay
Synap: Synaptogenesis
NOG: Neurite outgrowth
Prolif: Proliferation
AUC: Area under the curve

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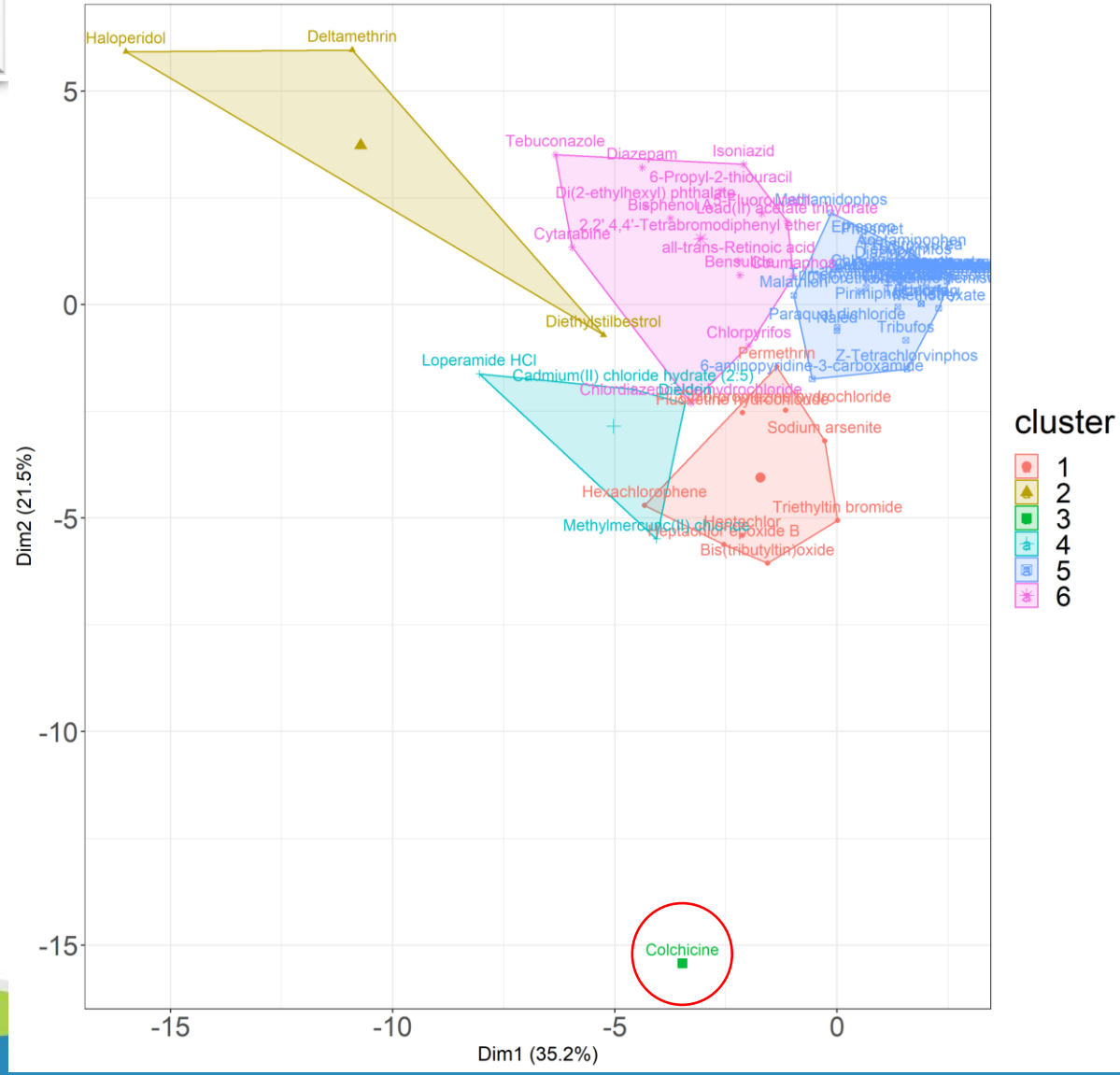
Evaluating ***selectivity*** is informative for identifying patterns of activity.

K-means clustering



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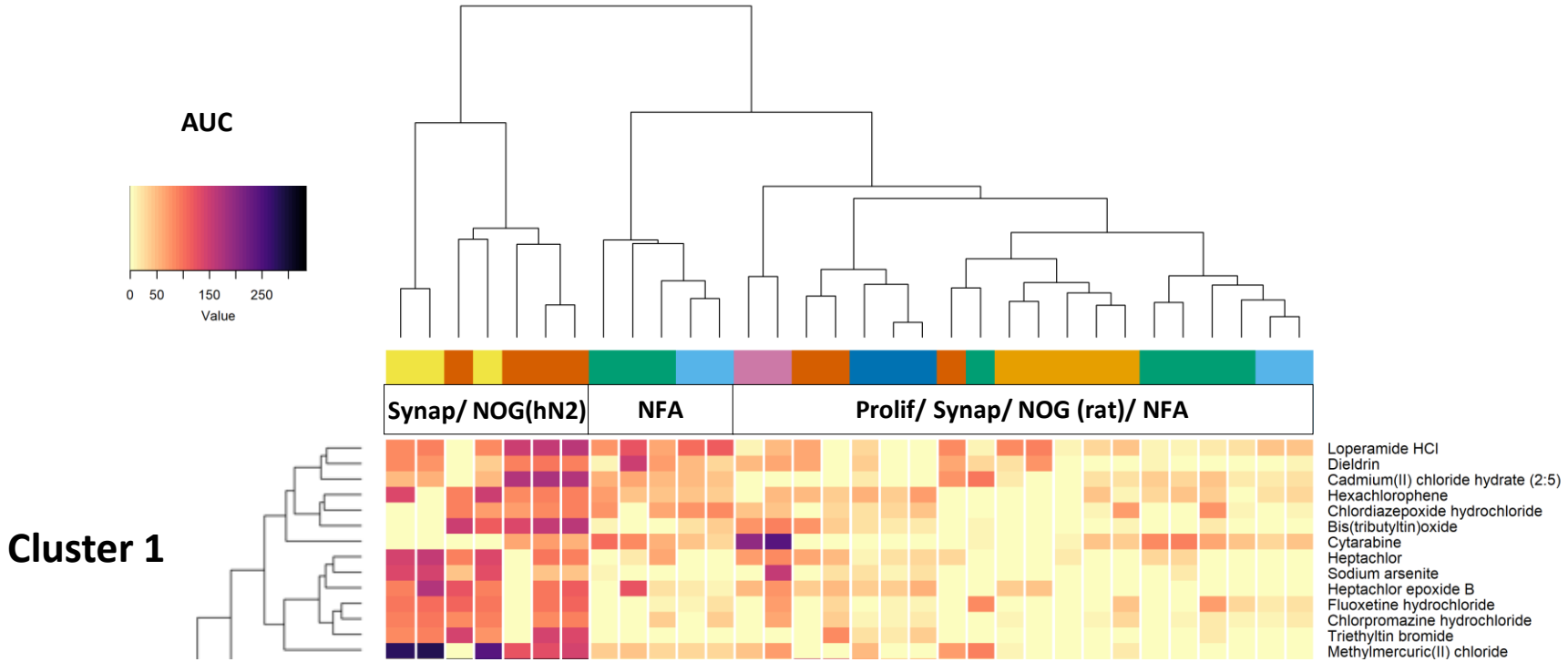
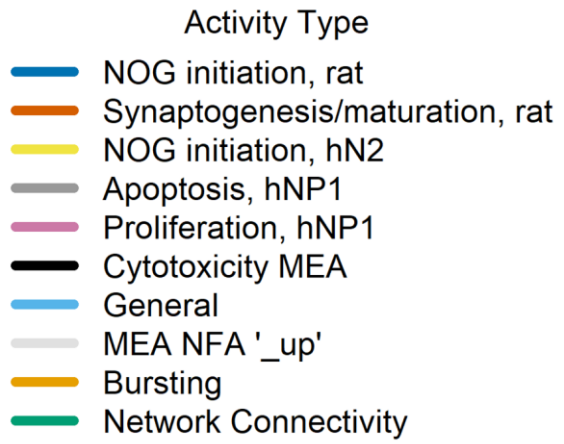
Evaluating ***selectivity*** is informative for identifying patterns of activity.





Cluster 1

High selectivity	Moderate/ Low selectivity
Synaptogenesis/ neurite maturation	Proliferation
NOG (hN2)	NOG (rat cortical)
	General neuronal activity/ network connectivity

NFA: Network formation assay
Synap: Synaptogenesis
NOG: Neurite outgrowth
Prolif: Proliferation
AUC: Area under the curve

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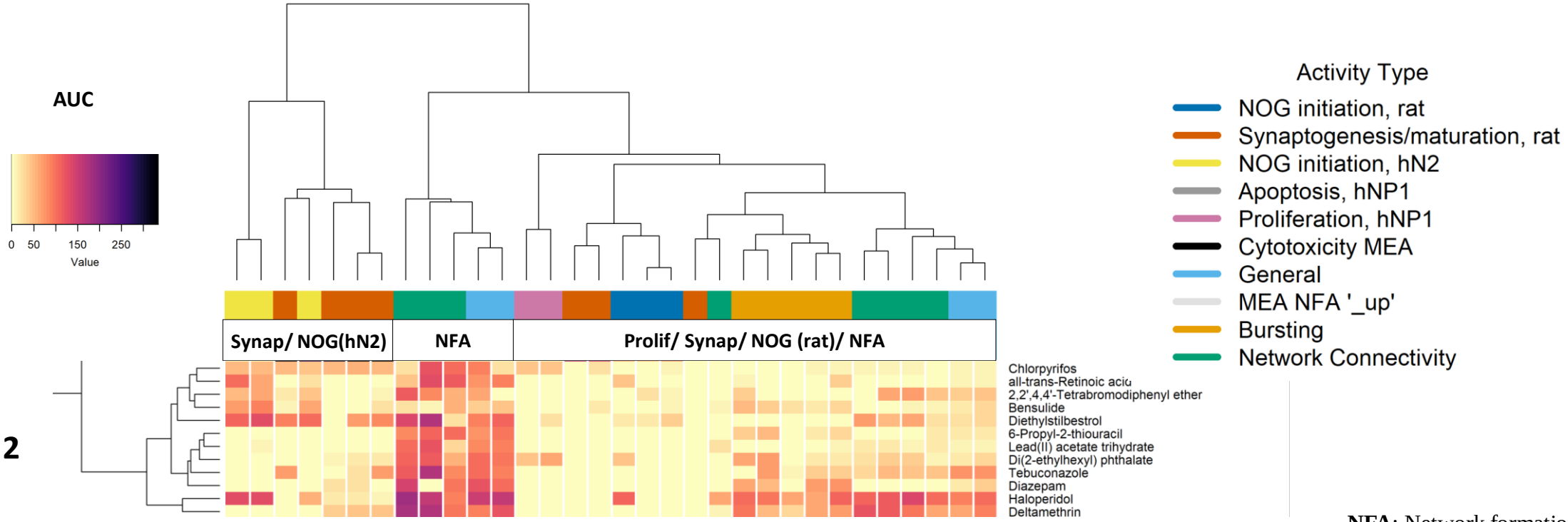

Cluster 2

High selectivity	Moderate/ Low selectivity
Network connectivity	NOG (hN2)
General neuronal activity	Bursting

NFA: Network formation assay
Synap: Synaptogenesis
NOG: Neurite outgrowth
Prolif: Proliferation
AUC: Area under the curve

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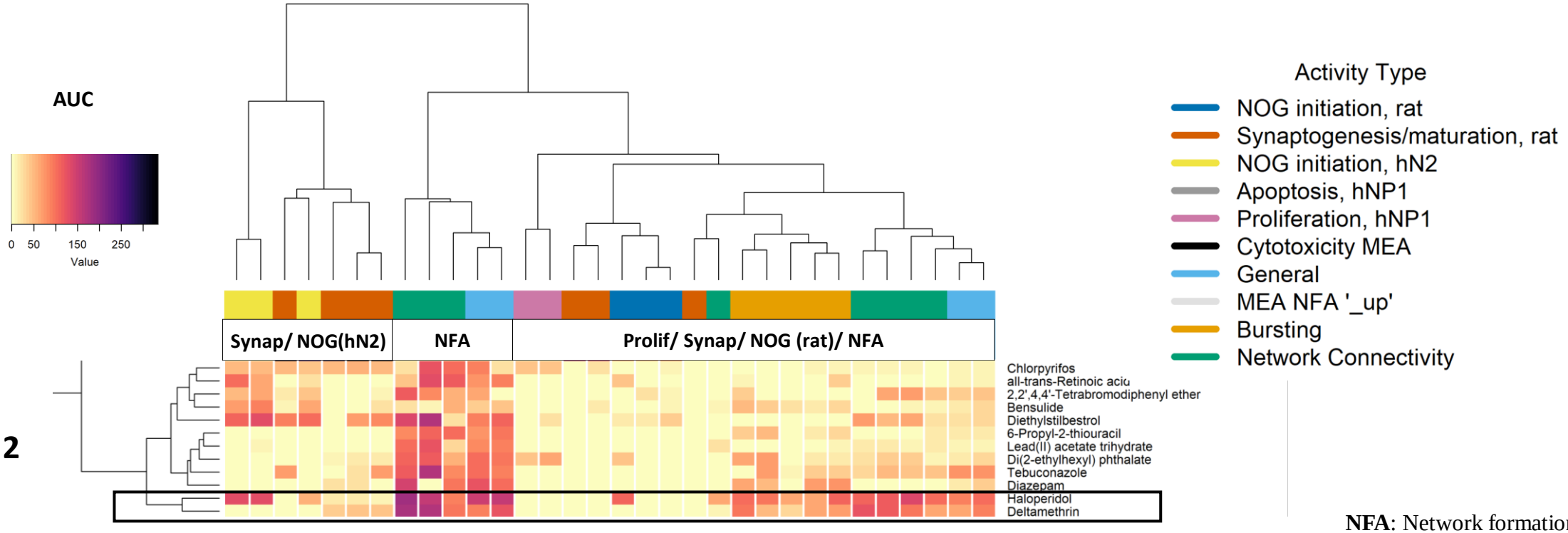
Evaluating ***selectivity*** is informative for identifying patterns of activity.



Cluster 2

Haloperidol: antipsychotic- Dopamine D_2 receptor antagonist

Deltamethrin: pyrethroid insecticide- voltage-gated sodium channels modulators

High selectivity	Moderate/ Low selectivity
Network connectivity	NOG (hN2)
General neuronal activity	Bursting

NFA: Network formation assay
Synap: Synaptogenesis
NOG: Neurite outgrowth
Prolif: Proliferation
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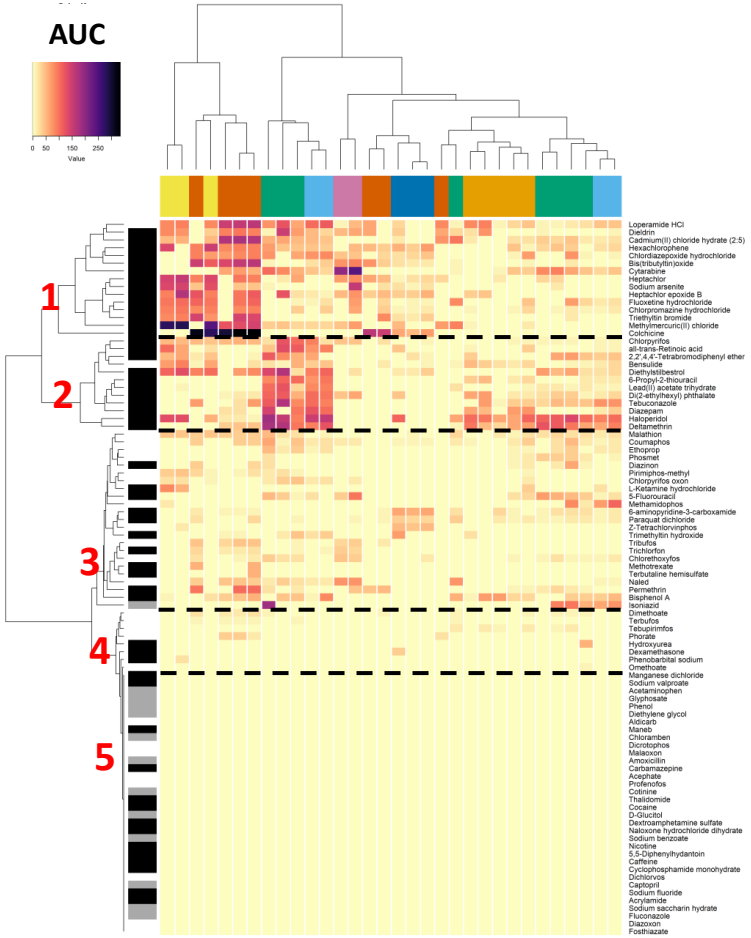
Evaluating ***selectivity*** is informative for identifying patterns of activity.



Key findings

- Selective data is more informative in identifying differential patterns of functional bioactivity compared to non-selective data.
- A subset of compounds demonstrate cell-type specific effects (active in the NOG assay in the hN2 cell model but not rat cortical).
- Selective activity clusters do not appear to be explained by shared mode-of-action.

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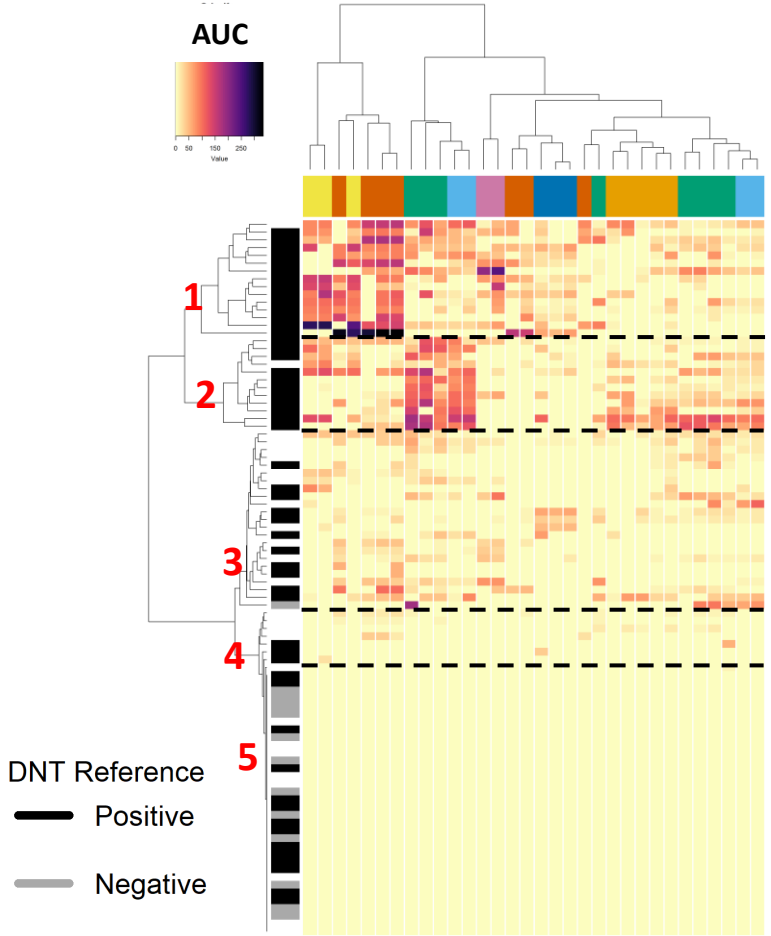
Can we build a model to classify compounds that demonstrate *in vivo* DNT bioactivity?



Classification		*In vivo* evaluation chemicals: Positive (53) Mundy et al. 2015, Aschner et al. 2016, Harrill et al. 2018	*In vivo* evaluation chemicals: Negative (13) Martin et al. *under revision*
	Cluster 1 Synap/ prolif/ NOG/ Neurite maturation	14	0
	Cluster 2 General/ network/ bursting activity/ synap	11	0
	Cluster 3 General/ network activity/ bursting/ synap/NOG	11	1
	Cluster 4 General/ network activity/ bursting/ synap/ NOG	3	0
	Cluster 5 'Inactive/ equivocal'	14	12

	Positive	Negatives
Selective activity (Clusters 1,2,3,4)	True positive: 39	False positive:1
Inactive/ equivocal (Cluster 5)	False negative: 14	True Negative: 12

Selective

Sensitivity= 74%

Specificity= 92%

Non-selective

Sensitivity= 93%

Specificity= 69%

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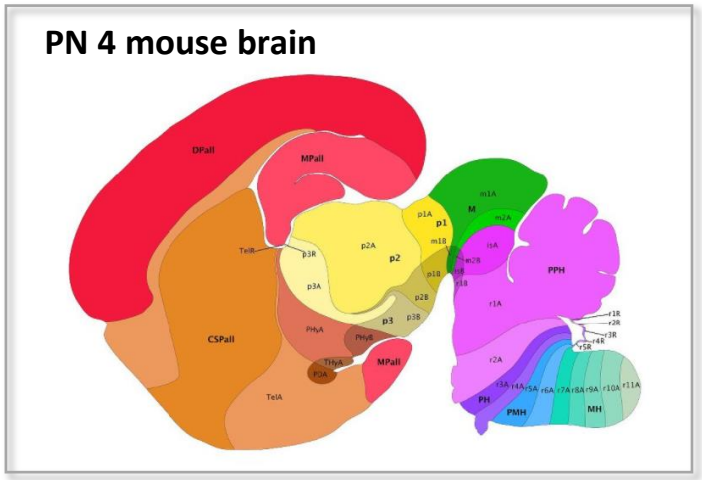
Can we identify biological gaps in the current EPA DNT NAM battery?

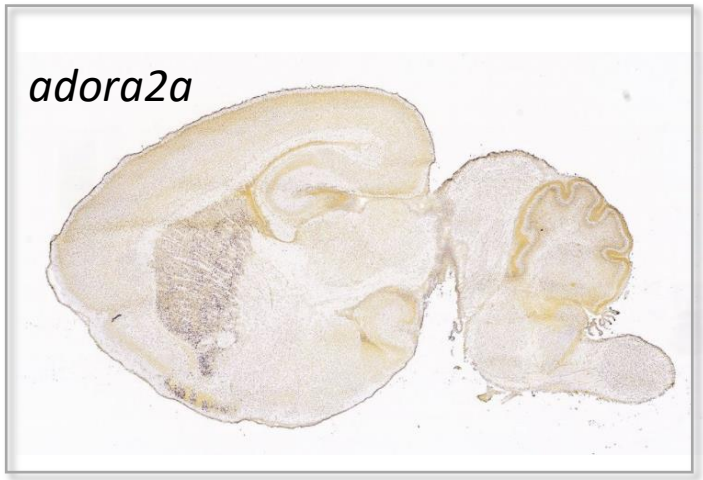
Are we capturing the target mechanism in the DNT NAM battery?

False negative: Caffeine*

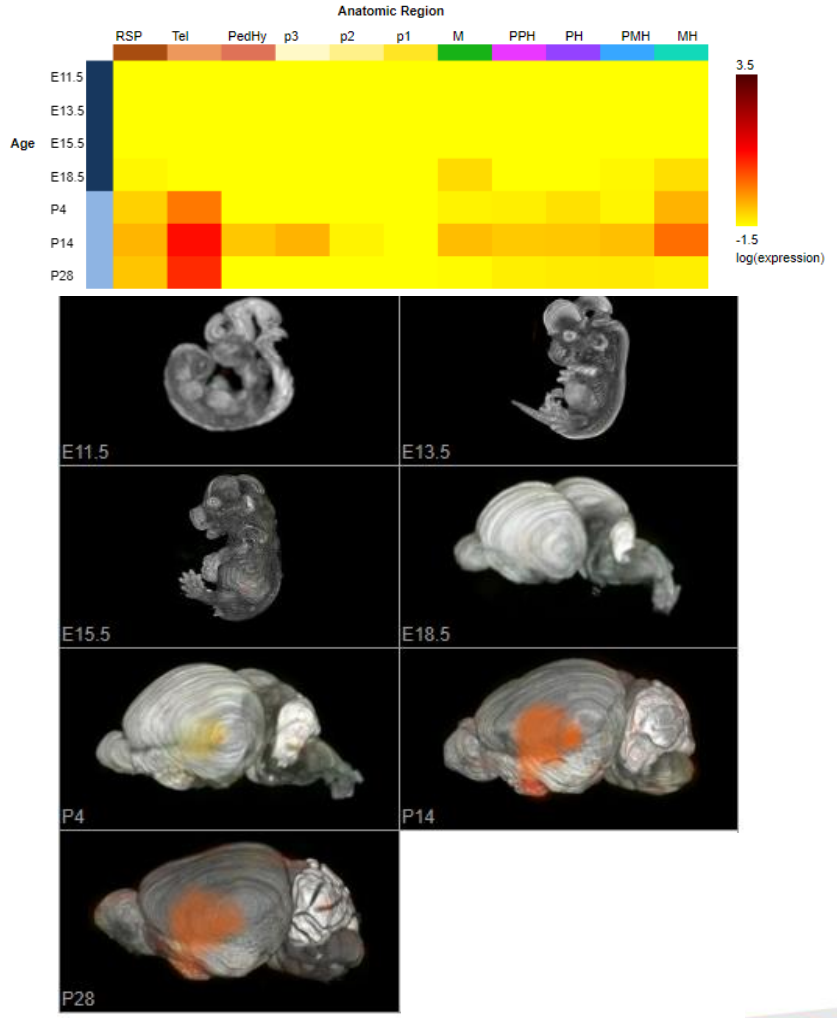
Caffeine targets adenosine receptor (adenosine A2a receptor)



https://developingmouse.brain-map.org/



In situ hybridization

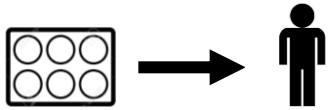


*Reference Mundy et al. (2015) for caffeine as a positive *in vivo* DNT evaluation chemical.

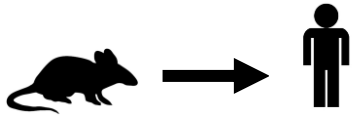
In vitro to *in vivo* extrapolation (IVIVE) using high-throughput toxicokinetic (HTTK) modeling

Are we testing at high enough concentrations *in vitro*?

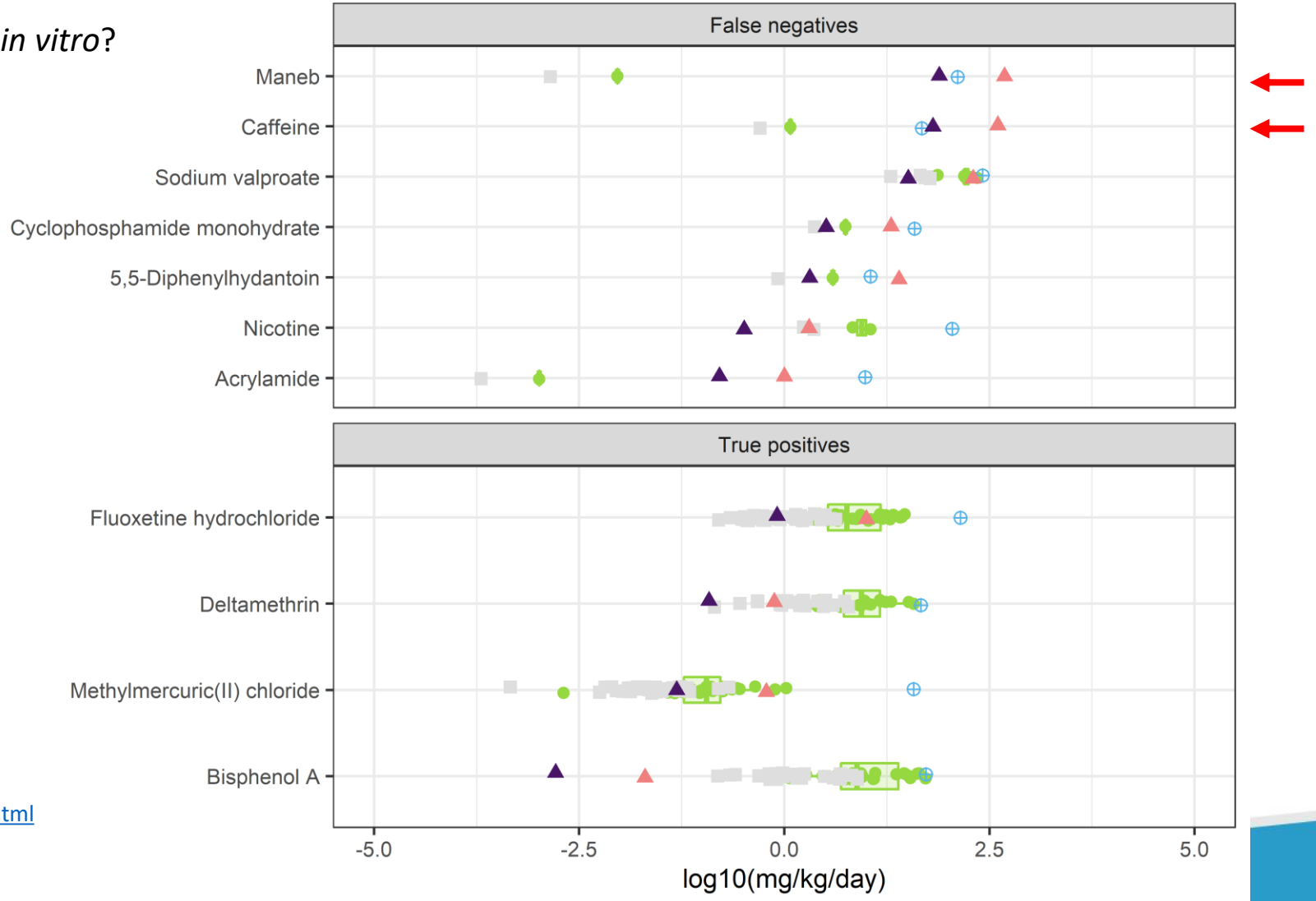
AED: administered equivalent dose



HED: human equivalent dose



- in vitro AED (human)
- in vitro max tested AED (human)
- in vivo dose (rodent)
- in vitro AED (95th percentile human)
- in vivo HED



'httk' R package: https://cran.r-project.org/web/packages/httk/index.html

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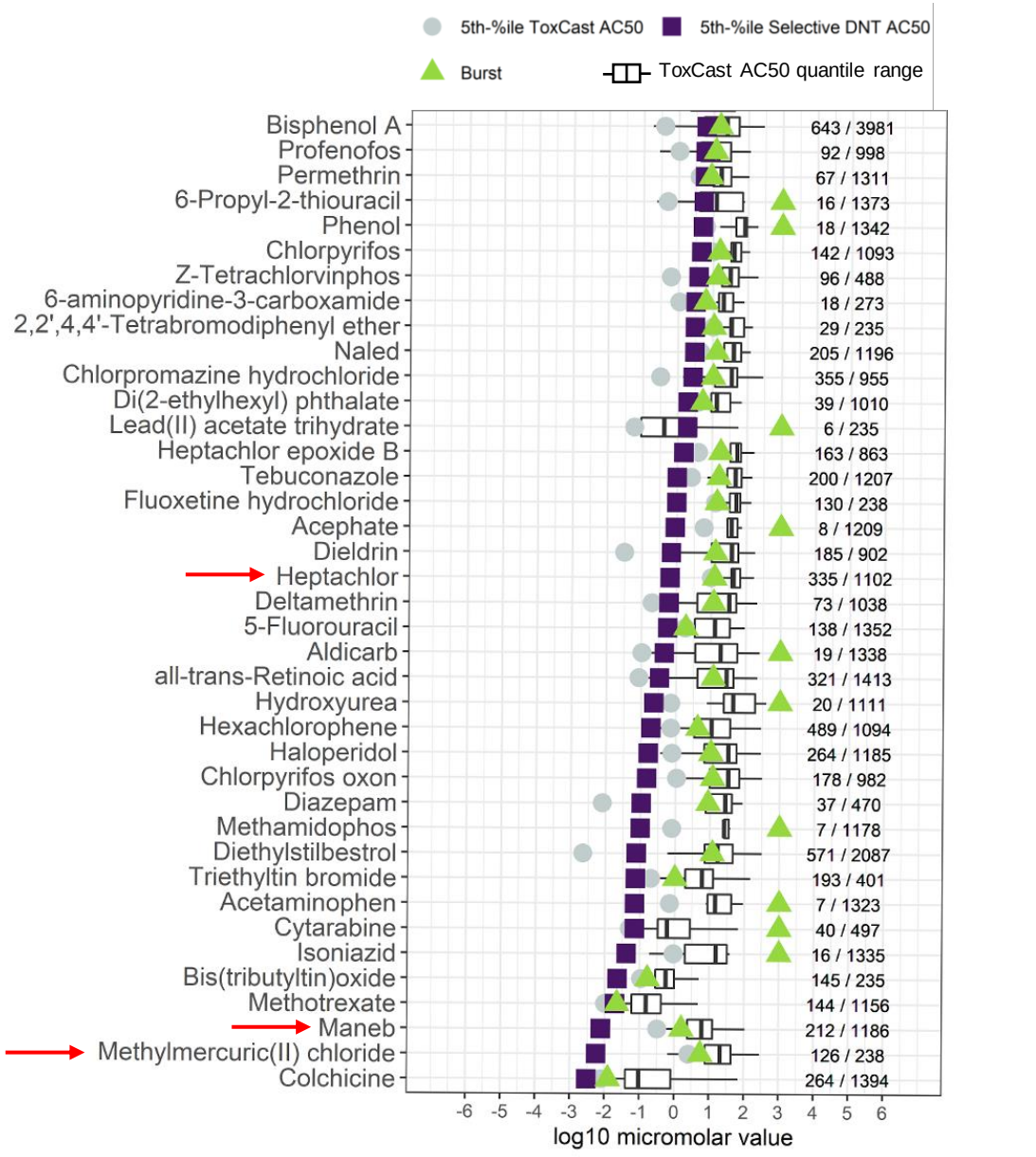
Comparison of *selective* DNT NAM activity to ToxCast/Tox21 database

ToxCast includes >**1,500 assay** endpoints and covers **heterogeneous** assay types, tissue sources, gene targets, and biological responses.

Examples of biological responses in ToxCast:

- Cell proliferation and death
- Cell differentiation
- Enzymatic activity
- Mitochondrial depolarization
- Protein stabilization
- Oxidative phosphorylation
- Reporter gene activation
- Receptor binding
- Receptor activity
- Metabolomic responses (stem cells)

https://comptox.epa.gov/dashboard/assay-endpoints



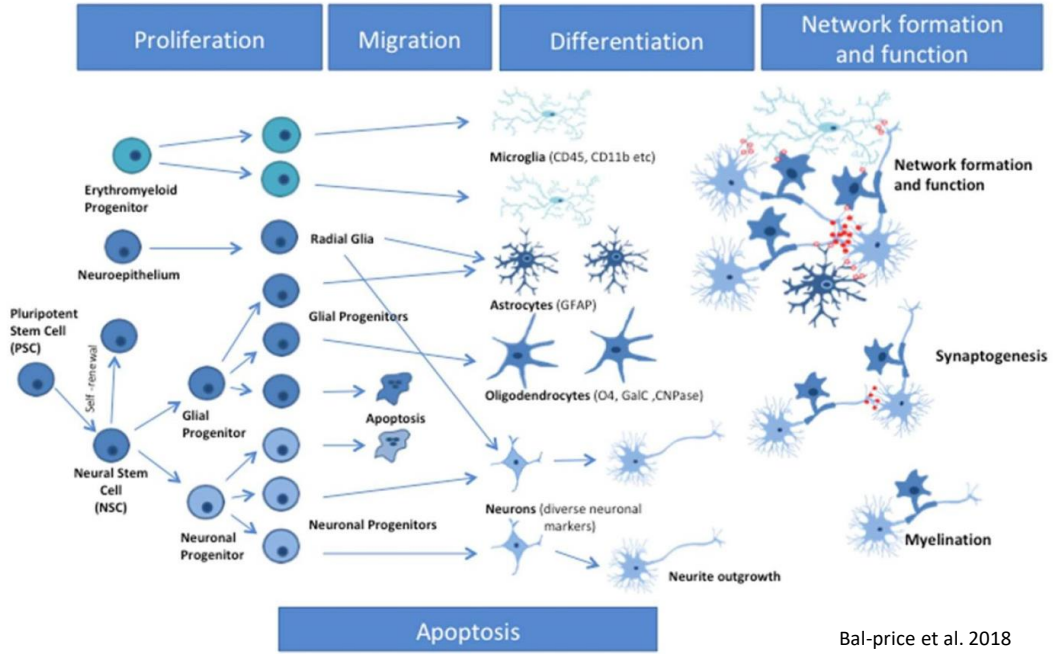
Conclusions

1) How does the DNT NAM battery collectively inform DNT-relevant bioactivity?

- Selective data is more informative in identifying differential patterns of functional bioactivity than non-selective data.
- Selective activity clusters do not necessarily appear to be explained by mode-of-action.

2) Can we build a model to classify compounds that demonstrate *in vivo* DNT bioactivity?

- Using the selectivity metric, DNT reference chemicals are classified with high specificity and moderate sensitivity.



Bal-price et al. 2018

3) Can we identify gaps in the current DNT NAM battery and/or broader ToxCast/ Tox21 database?

- False negatives provide insight into experimental and biological limitations which may be associated with cell-type, species or developmental timepoint.
- DNT NAMs data provides added value to ToxCast/ Tox21 database from the perspective of capturing health protective potencies.

Questions?

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Amy Carpenter
Seline Choo
Jackson Keever
Megan Culbreth

Contact Info:

Kelly Carstens, PhD
U.S. Environmental Protection Agency
Research Triangle Park, NC

Email: carstens.kelly@epa.gov
Office: 919-541-3834

Assay data:
Available in ToxCast invitrodb v 3.4
https://doi.org/10.23645/epacomptox.6062479.v6