Computational approaches to integrate DNT NAMs for fit-for-purpose identification of DNT hazard

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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.

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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.
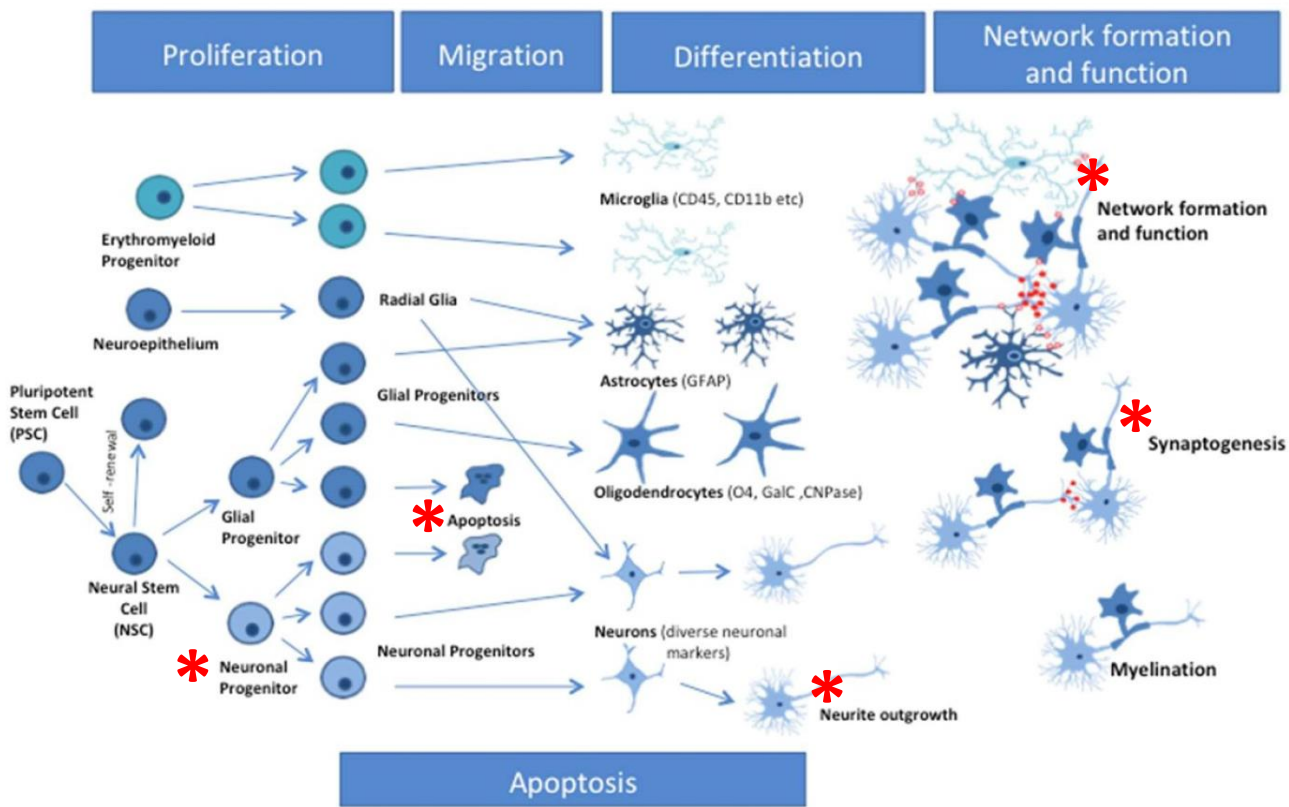- Some neural cell-types may be differentially sensitive to perturbation.

Key functional processes guiding human and rodent cortical development
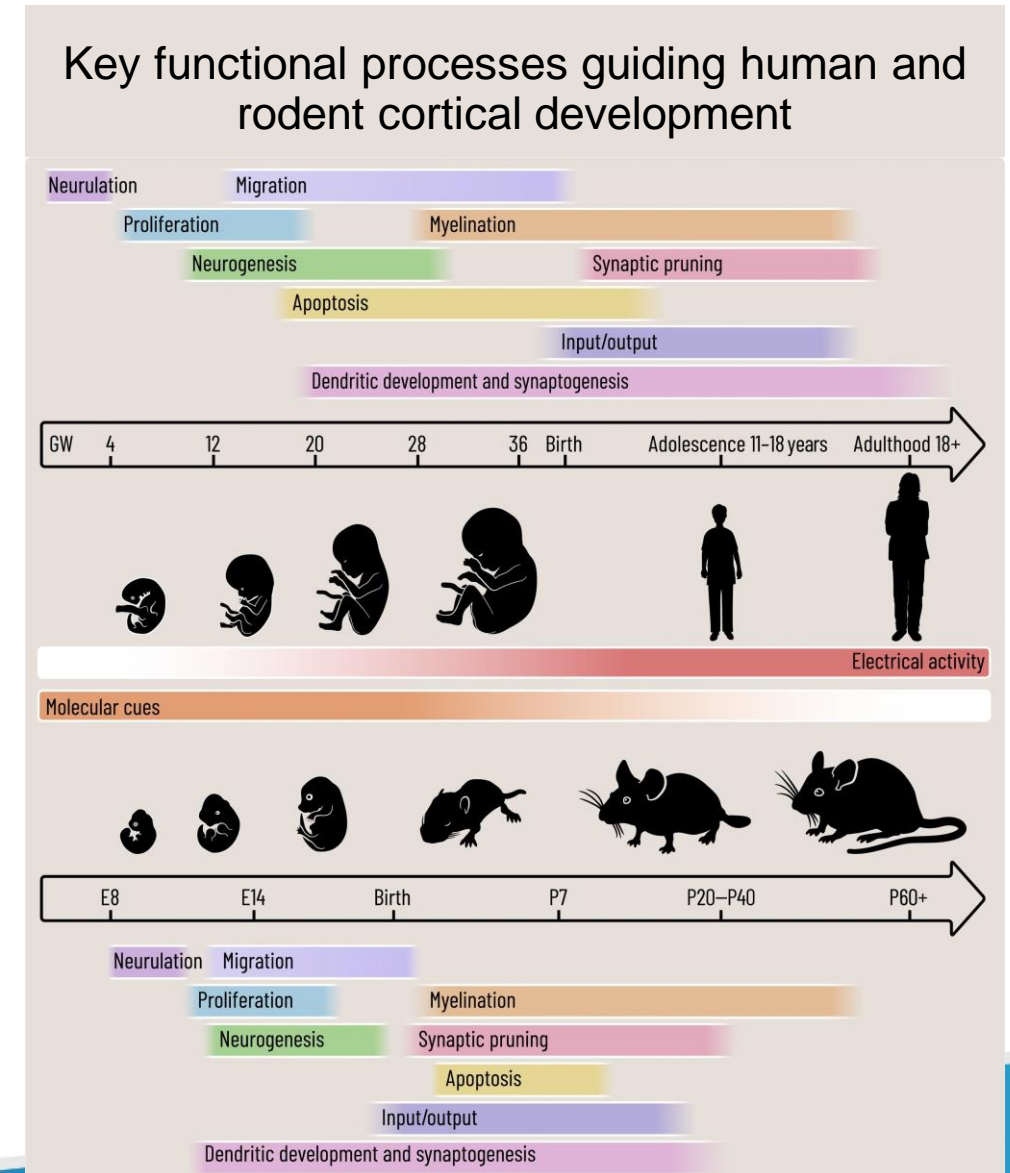
Chini and Hanganu-Opatz. 2021. Trends in Neuro.

Overview

DNT NAMs: multi-dimensional DNT screening assays that cover complex neurobiological space: multiple 'key events' in neurodevelopment, different cell-types, developmental timepoints, and species.

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

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

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

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)

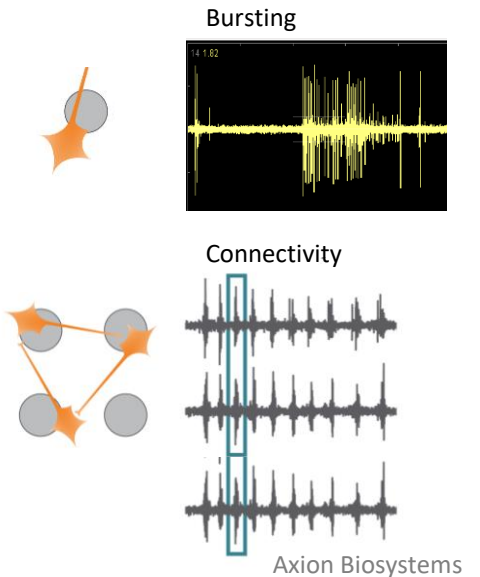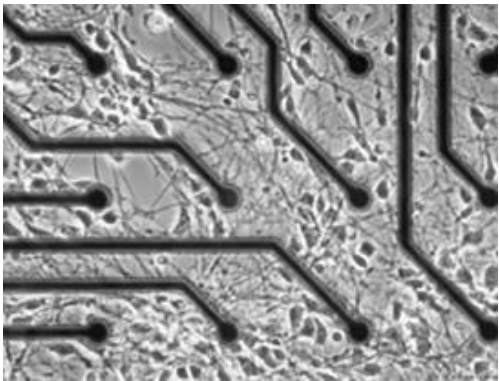Sachana, M., et.al. 2019, Toxicological Sciences

Experimental models in the EPA DNT NAM battery

Microelectrode Array (MEA)
Network Formation Assay (NFA)

← 92 chemicals →

High Content Imaging

48-well culture plate
16 electrodes per well

Bursting

Connectivity

Axion Biosystems

96-well culture plate
Immunohistochemistry
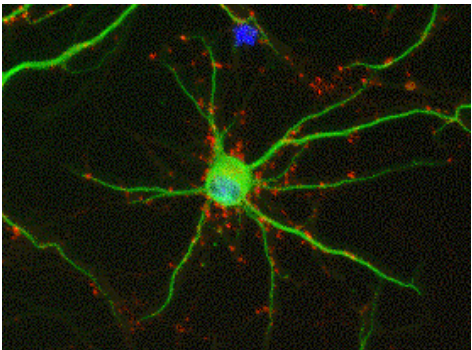
Image Analysis

Cell culture	Activity type	# endpoints
Primary rat cortical neurons (DIV 5, 7, 9, 12)	↓↑ General activity	4
	↓↑ Network connectivity	8
	↓↑ Bursting	5
	Cytotoxicity	2
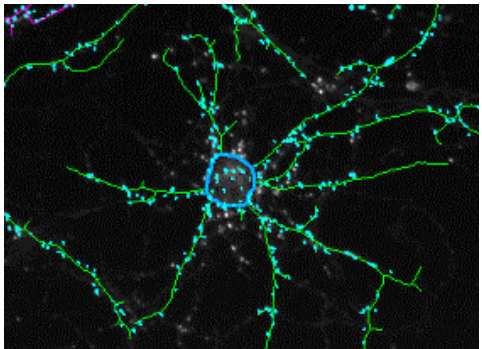
Cell culture	Assays/ Key events	# endpoints
Primary rat cortical neurons	Neurite Outgrowth (NOG)	4
	Synaptogenesis and Neurite maturation	8
Human hN2 neural cells	NOG	4
Human hNP1 neuroprogenitors	Proliferation	3
	Apoptosis	2

Defining bioactivity using the ToxCast pipeline
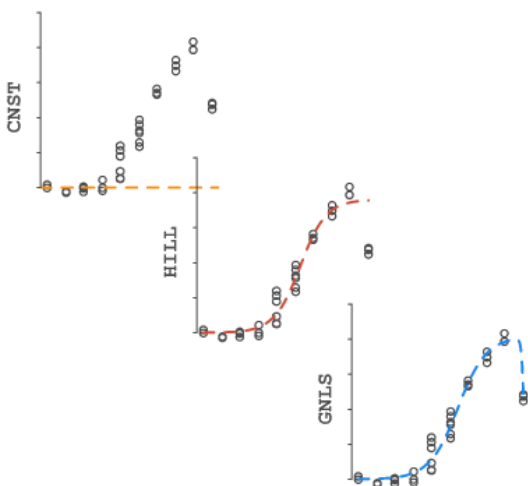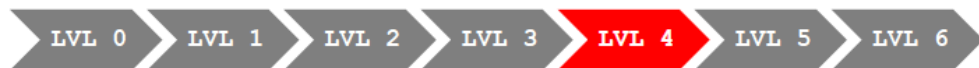
Model fitting (constant, hill, gain-loss)

Select winning model and hit-calling

$\mu_i = 0$

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

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

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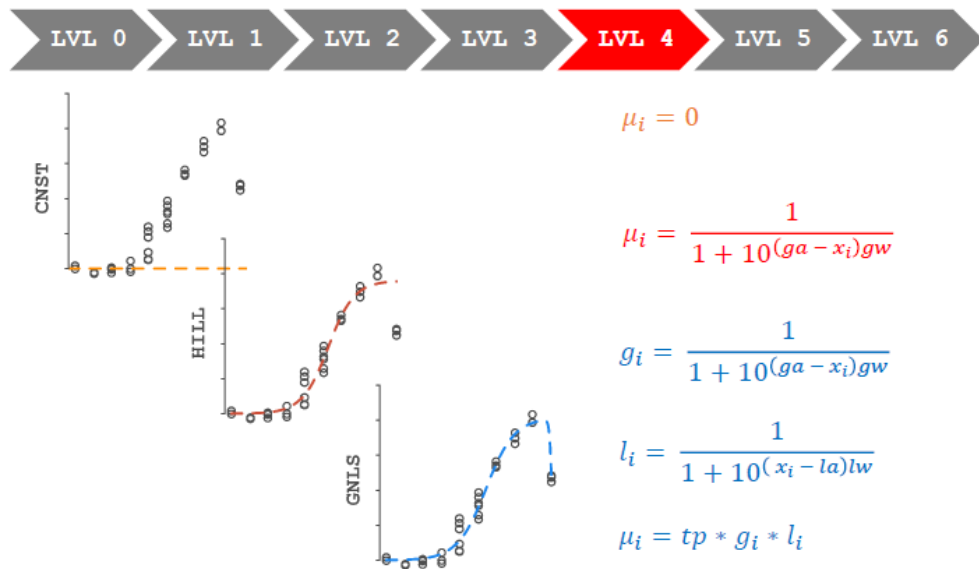
$$\mu_i = tp * g_i * l_i$$

Point of departure estimates:

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

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

Defining bioactivity using the ToxCast pipeline

Model fitting (constant, hill, gain-loss)

Select winning model and hit-calling

LVL 0 → LVL 1 → LVL 2 → LVL 3 → **LVL 4** → LVL 5 → LVL 6

CNST

HILL

GNLS

$$\mu_i = 0$$

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

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

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

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

CCTE_Shafer_MEA_dev_bursting_electrodes_number_dn

Percent Activity

Concentration (µM)
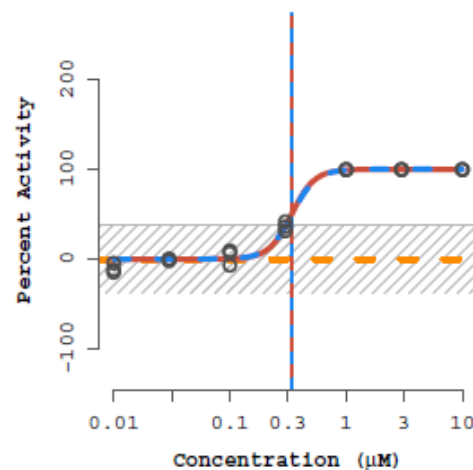
```
ASSAY:     AEID2500 (CCTE_Shafer_MEA_dev_bursting_electro

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:        1.29      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: 38.3   HIT-CALL: 1    FITC: 41    ACTP: 1

FLAGS:
```
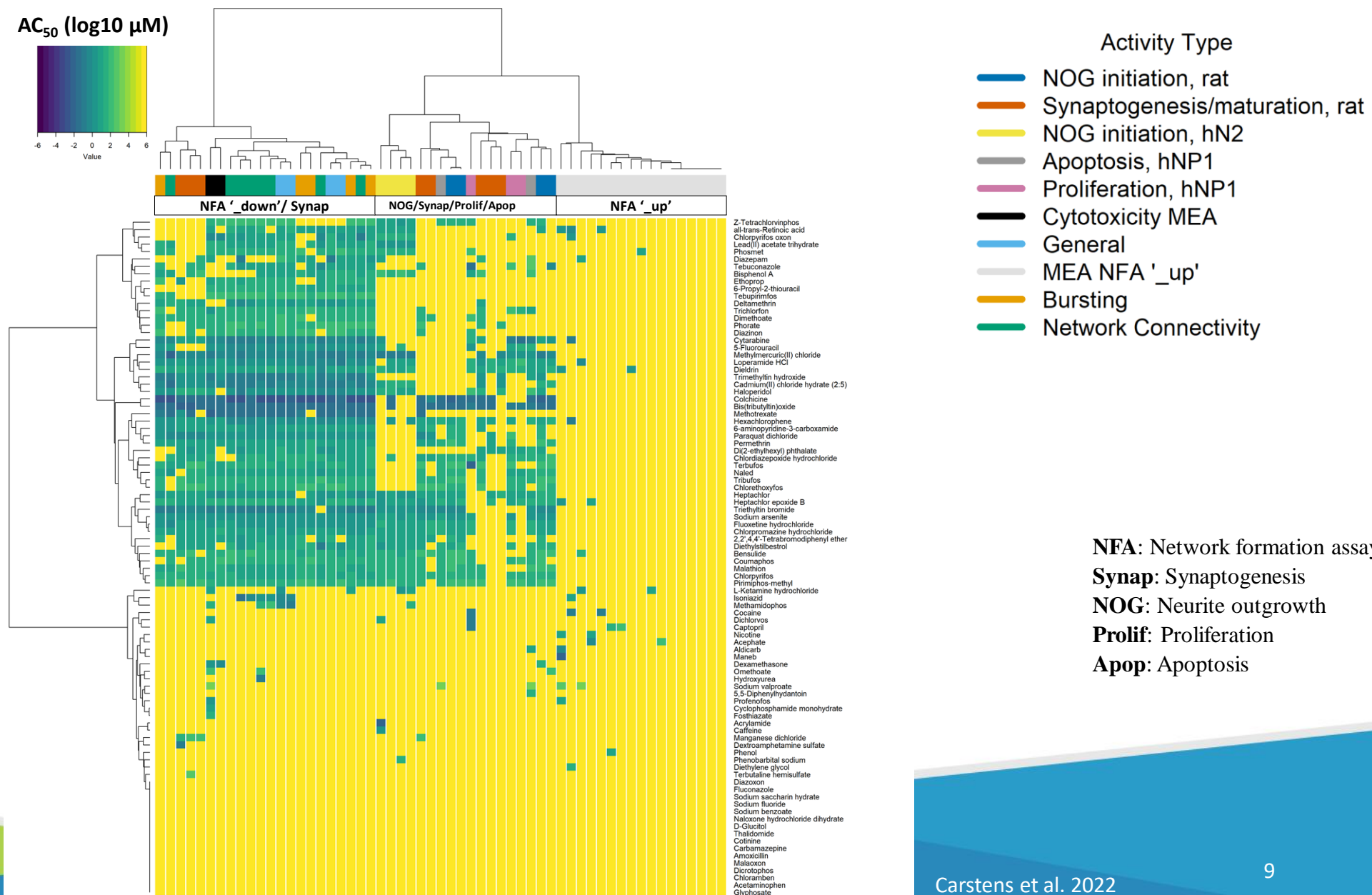
ToxCast pipeline (tcpl) R package (version 2.0.3 publicly available) (Filer et al. 2017)
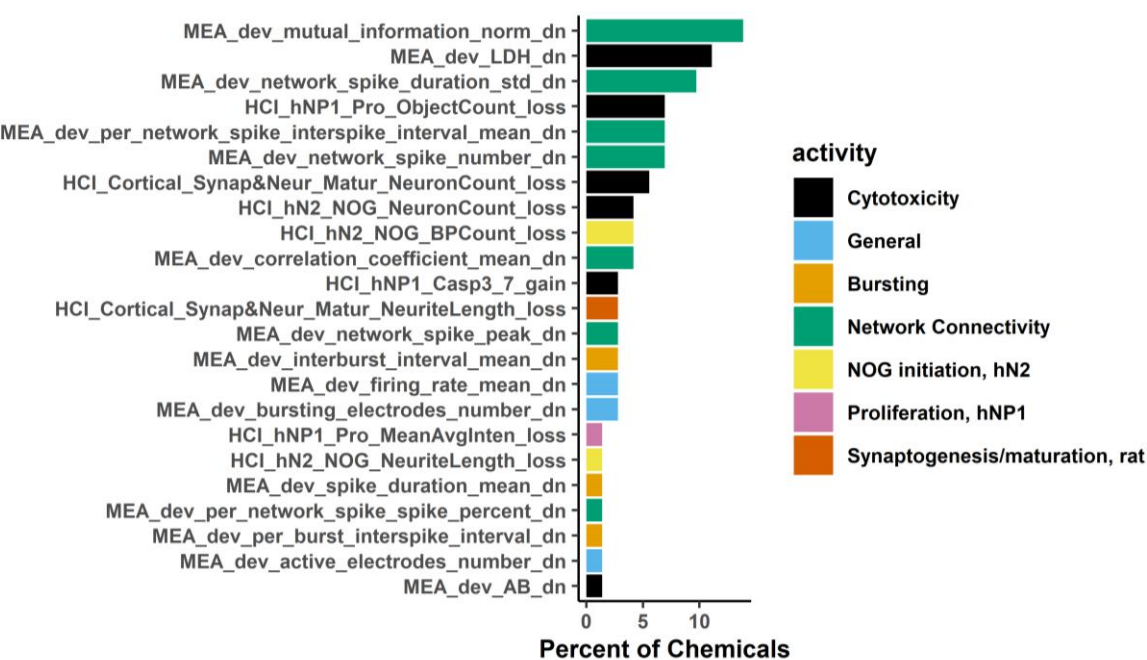
https://cran.r-project.org/web/packages/tcpl/vignettes/Data_processing.html#level-4

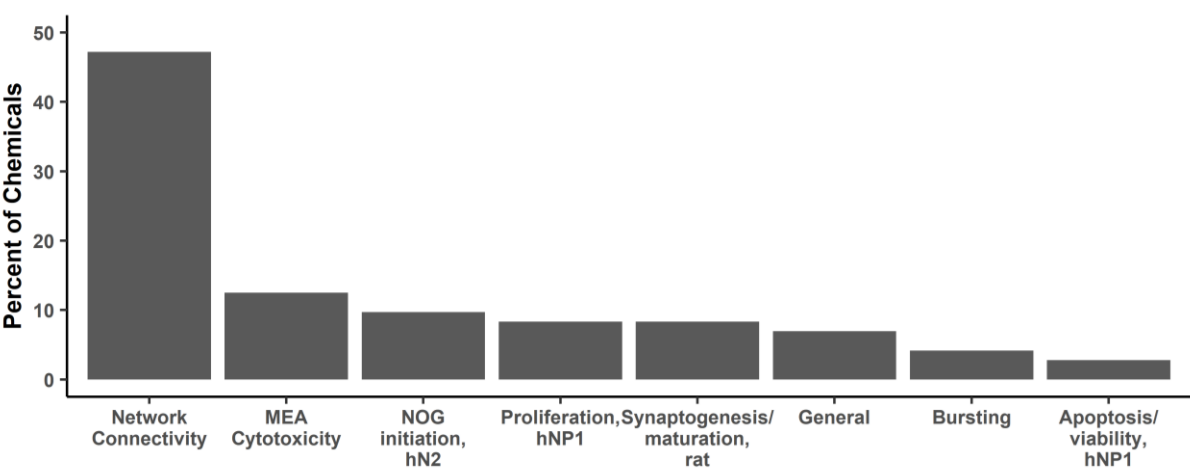# How does a broad screening battery collectively inform DNT-relevant bioactivity?

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

Carstens et al. 2022

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Evaluating sensitivity in the DNT NAM battery

Minimum potency by endpoint

Minimum potency by activity type

Network connectivity and cytotoxicity are the most sensitive 'activity types' in the battery.

Evaluating sensitivity in the DNT NAM battery

Feature importance

A supervised machine learning algorithm identifies cytotoxicity (hNP1 cell line) as the most important endpoint in predicting minimum potency in the DNT NAM battery.

$$randomForest(minimum.potency \sim + potency.matrix)$$

%IncMSE: Percent increase in mean square error (MSE)

Calculating a *selectivity* metric:

Selectivity: activity at concentrations lower than cytotoxicity.
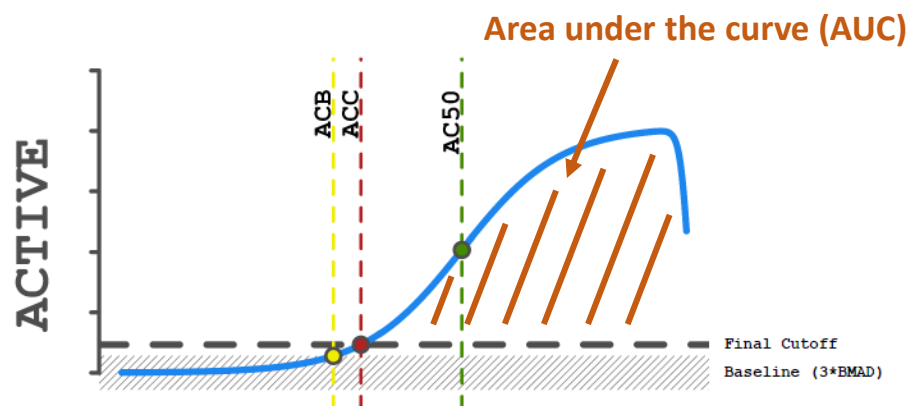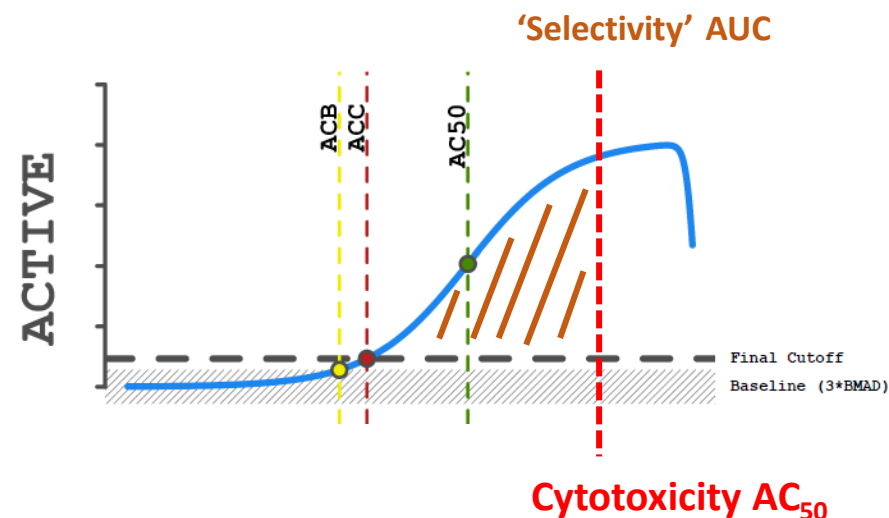
https://cran.r-project.org/web/packages/tcpl/vignettes/Data_processing.html#level-4

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

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

Carstens et al. 2022

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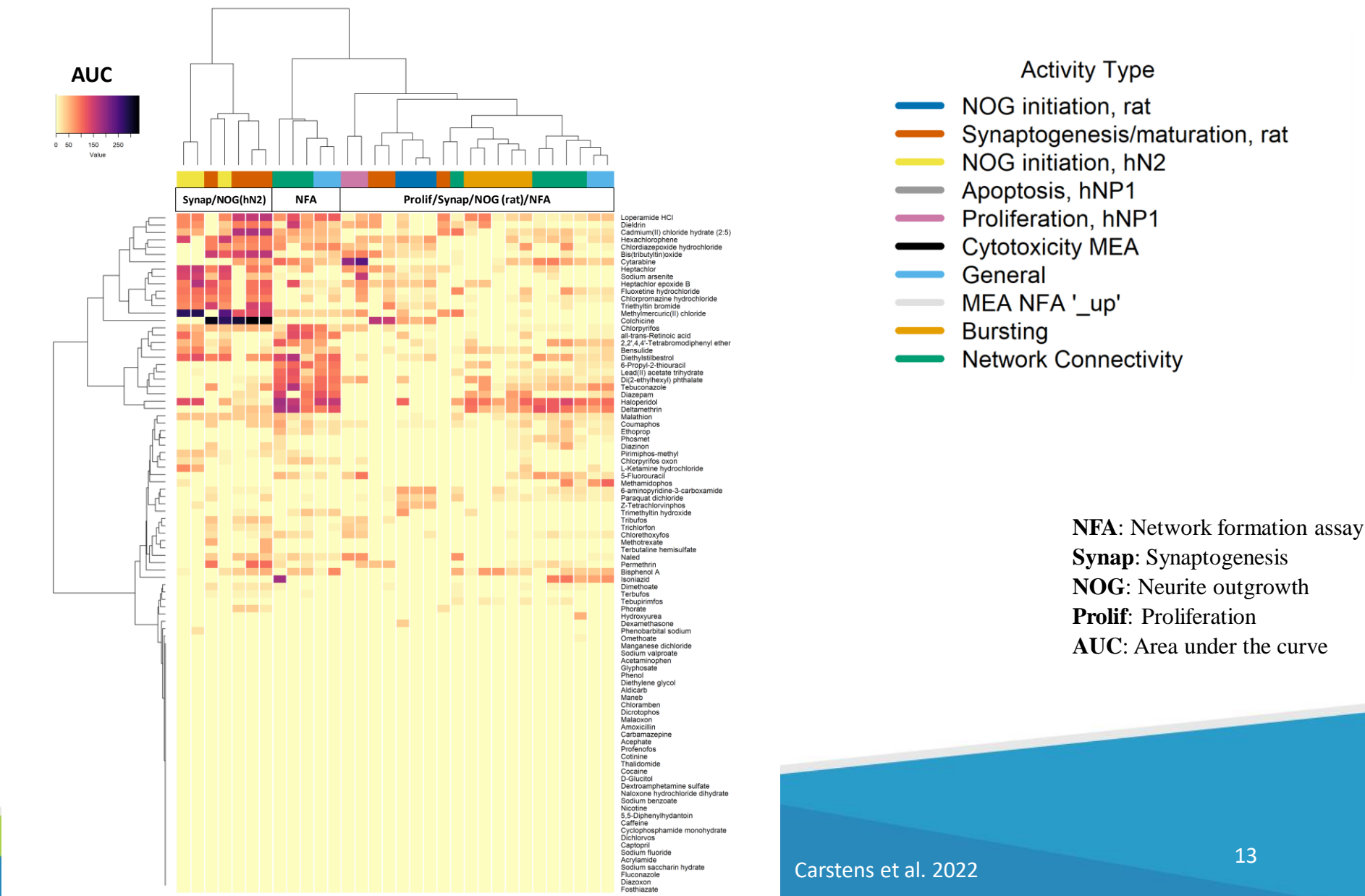
Evaluating *selectivity* is informative for identifying patterns of activity.

Unsupervised machine learning algorithm
K-means clustering
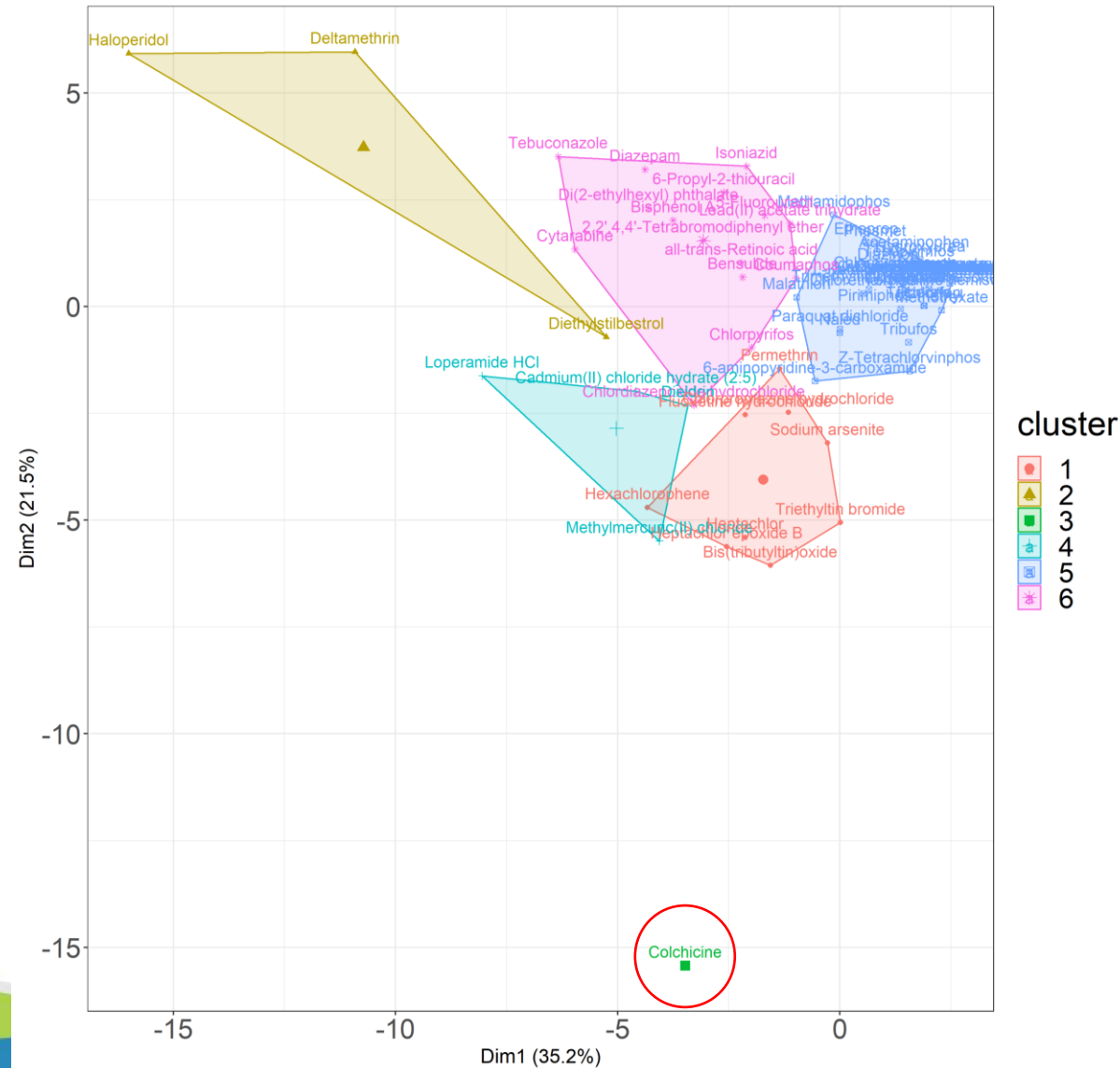
Evaluating *selectivity* is informative for identifying patterns of activity.
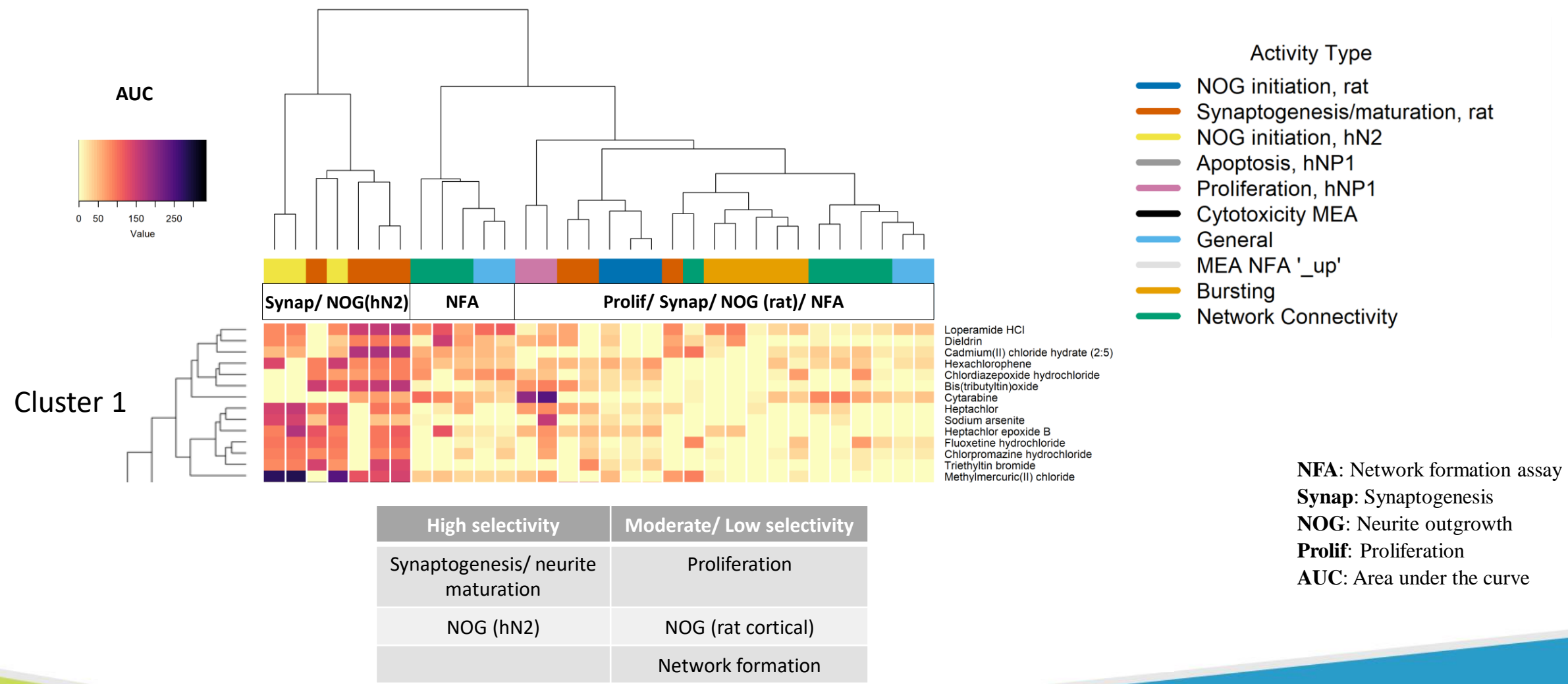
Carstens et al. 2022

High selectivity	Moderate/ Low selectivity
Synaptogenesis/ neurite maturation	Proliferation
NOG (hN2)	NOG (rat cortical)
	Network formation

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

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

Cluster 2
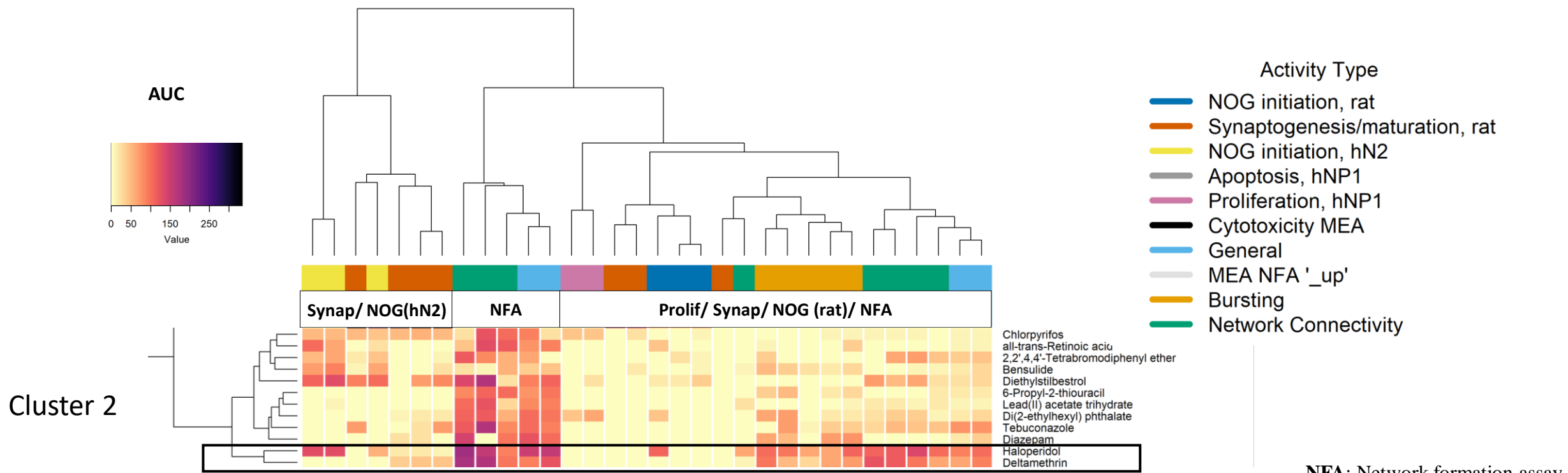
Haloperidol: antipsychotic- Dopamine D_2 receptor antagonist

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
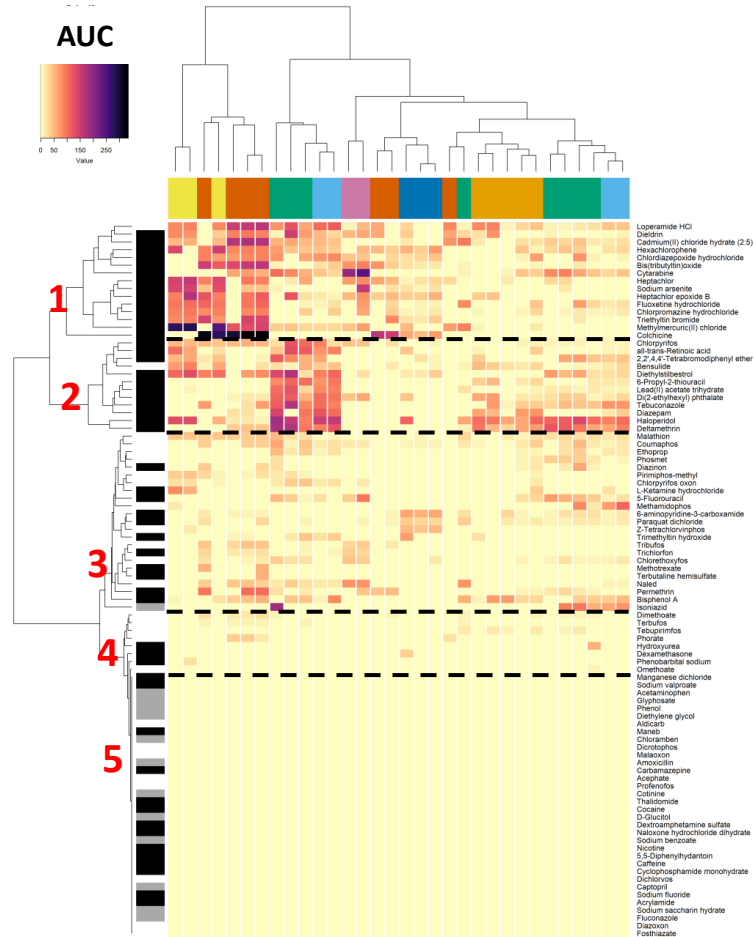**AUC**: Area under the curve

Carstens et al. 2022

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.

- Limitations in evaluating relationships between bioactivity clusters and mode-of-action or activity-structure due to a limited dataset.

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Can we build a model to classify compounds that demonstrate *in vivo* DNT bioactivity?
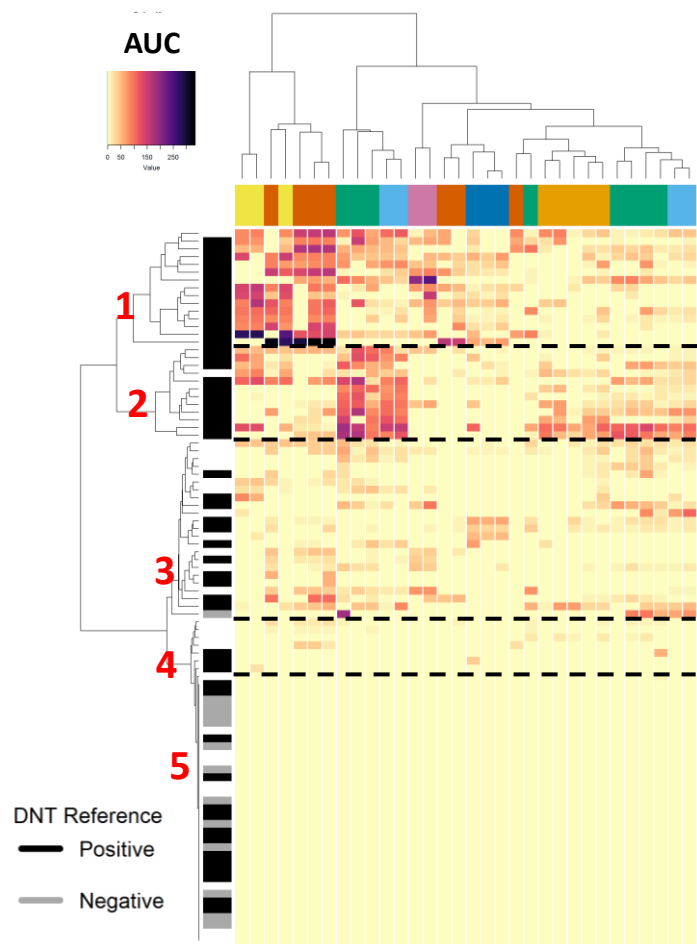
		In vivo evaluation chemicals	
		Positive (53) Mundy et al. 2015 Aschner et al. 2016 Harrill et al. 2018	**Negative (13)** Martin et al. *under revision*
Classification	**Cluster 1** Synap/ prolif/ NOG/ Neurite maturation	14	0
	Cluster 2 General/ network/ bursting activity/ synap	11	0
	Cluster 3 General/ network activity/ bursting/ synap/NOG	11	1
	Cluster 4 General/ network activity/ bursting/ synap/ NOG	3	0
	Cluster 5 'Inactive/ equivocal'	14	12

AUC

DNT Reference
— Positive
— Negative

	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%

Carstens et al. 2022
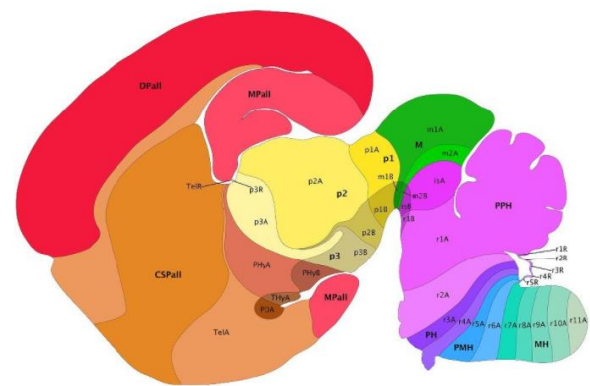
Can we identify biological gaps in the current EPA DNT NAM battery?

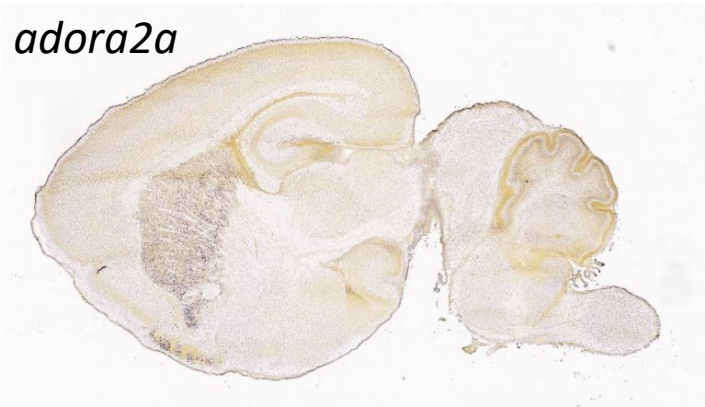**Are we capturing the target mechanism in the DNT NAM battery?**

False negative: Caffeine

Caffeine targets adenosine receptor (adenosine A2a receptor)
Is adenosine expressed in cortical cells in early development?

P4 mouse brain

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

adora2a

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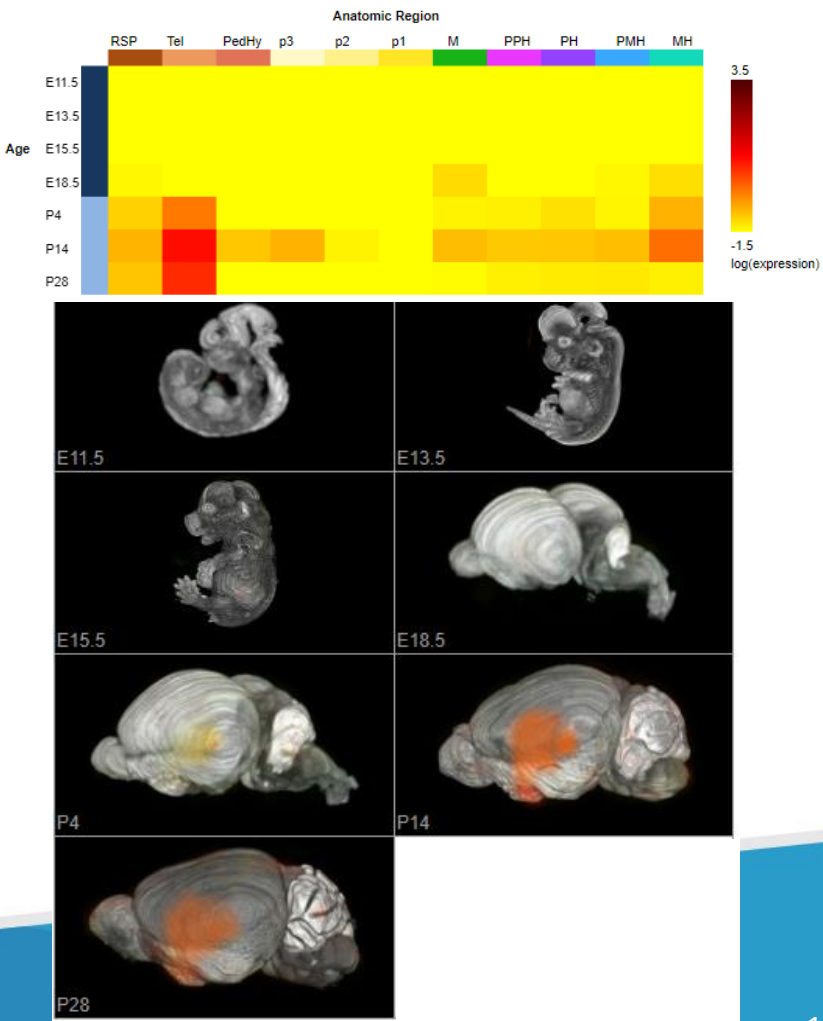
In vitro to *in vivo* extrapolation (IVIVE) using high-throughput toxicokinetic (HTTK) modeling

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

AED: administered equivalent dose
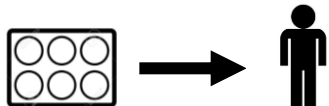
HED: human equivalent dose

Legend:
- ● 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

Carstens et al. 2022
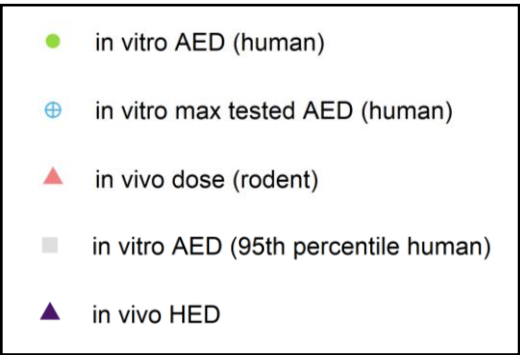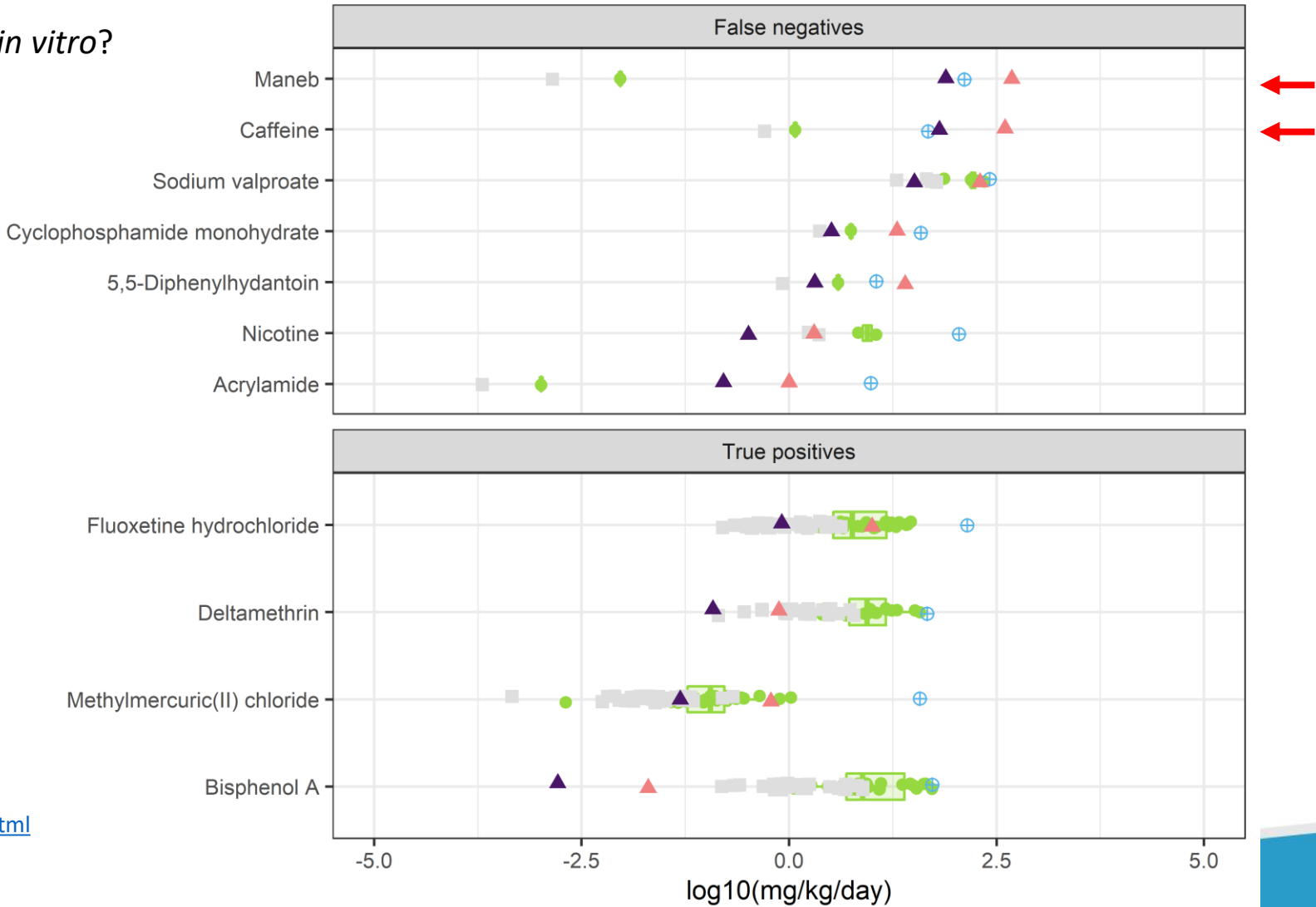
Identifying gaps in the EPA DNT NAM battery

Is a false negative active in other DNT NAMs that are not included in the current EPA battery?

False negative: Maneb

Active in 3 DNT NAM endpoints outside the US EPA battery

➢ UKN4: Neurite area in LUHMES cells (human dopaminergic precursor)
➢ UKN5: Neurite area in peripheral nervous system (human derived dorsal root ganglia)
➢ UKN2: Neural crest cell (NCC) migration assay (human derived)

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

Process	Assays	References
*Proliferation	→ hNP1 NPC1	Harrill *et al.* (2018) Baumann *et al.* (2016) and Barenys *et al.* (2017)
	UKN1	Balmer *et al.* (2012)
*Apoptosis Migration	→ hNP1 NPC2	Harrill *et al.* (2018) Baumann *et al.* (2016) and Barenys *et al.* (2017)
Neuron differentiation	UKN2 NPC3	Nyffeler *et al.* (2017) 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) UKN 4 & 5 (rat) NPC4	Harrill *et al.* (2018) Krug *et al.* (2013) 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

Results from the 2020 EFSA Scientific Report on DNT NAMs (µM)

compound name	CAS #	Selection criteria	UKN4 neurite area	UKN5 peripheral neurite area	NPC4 neurite length	NPC4 neurite area	UKN2 (NCC migration)	NPC2a72h (radial glia migr.)	NPC2a120h (radial glia migr.)	NPC2b (neuron migration)	NPC2c (oligo. migr.)
Maneb	12427-38-2	in vivo positive	3.2	2	no hit	no hit	11.4	no hit	no hit	no hit	no hit

Modified from Masjothusmann et al. 2020

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
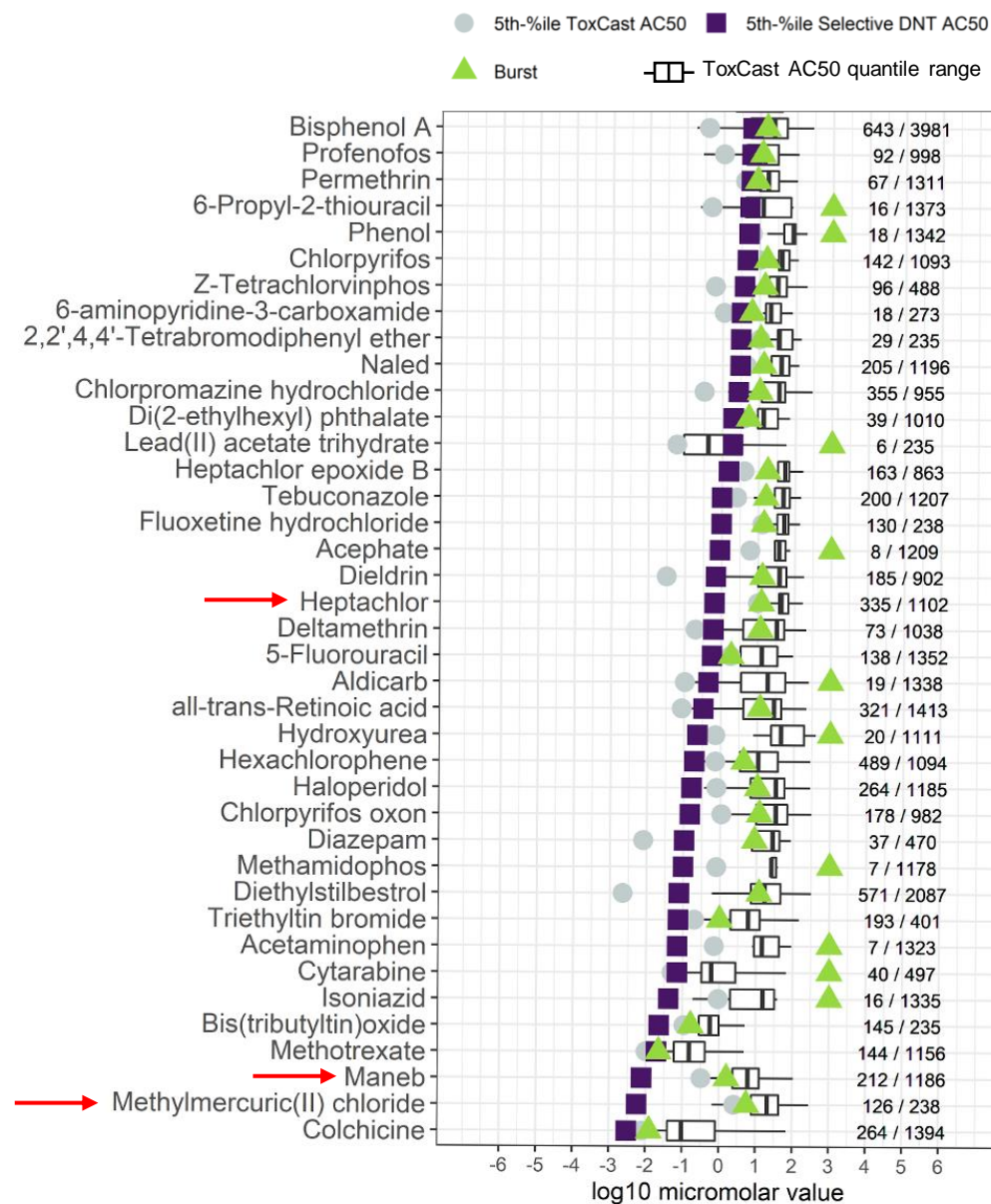
Conclusions

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 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.

- False negatives provide insight into experimental and biological limitations.

Bal-price et al. 2018

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

- Identified gaps in target receptor which may be associated with cell-type, species or developmental timepoint.

- Identified gaps in the current battery of DNT NAMs that appear to be covered by assays representing additional 'key events' in neurodevelopment.

- DNT NAMs data provides added value to ToxCast/ Tox21 database from the perspective of capturing health protective potencies.
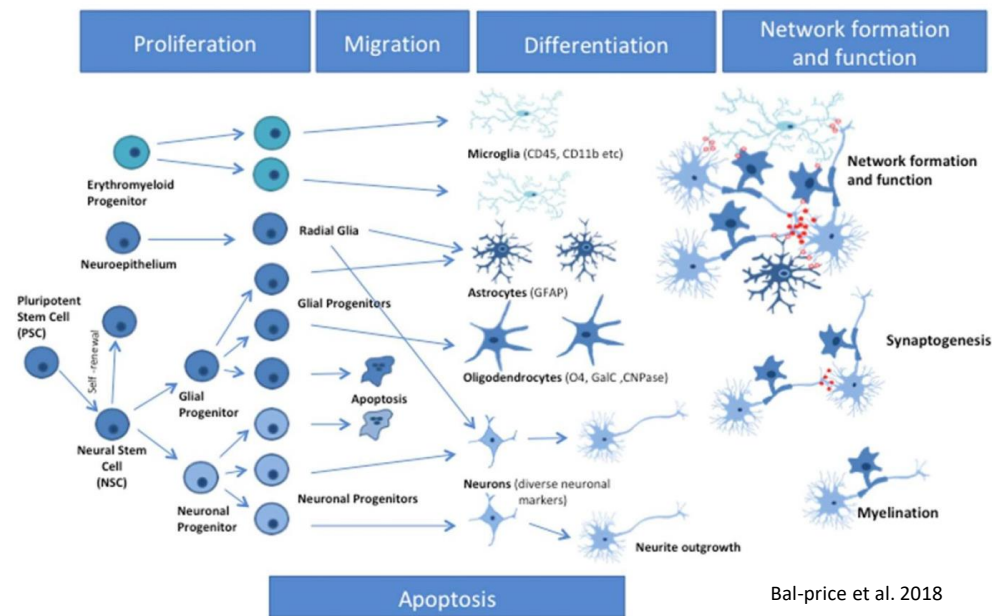
Questions?

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Cina Mack
Melissa Martin
Amy Carpenter
Seline Choo
Jackson Keever
Josh Harrill
Megan Culbreth

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Office: 919-541-3834

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

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Biomolecular and Computational Toxicology Division
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