



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

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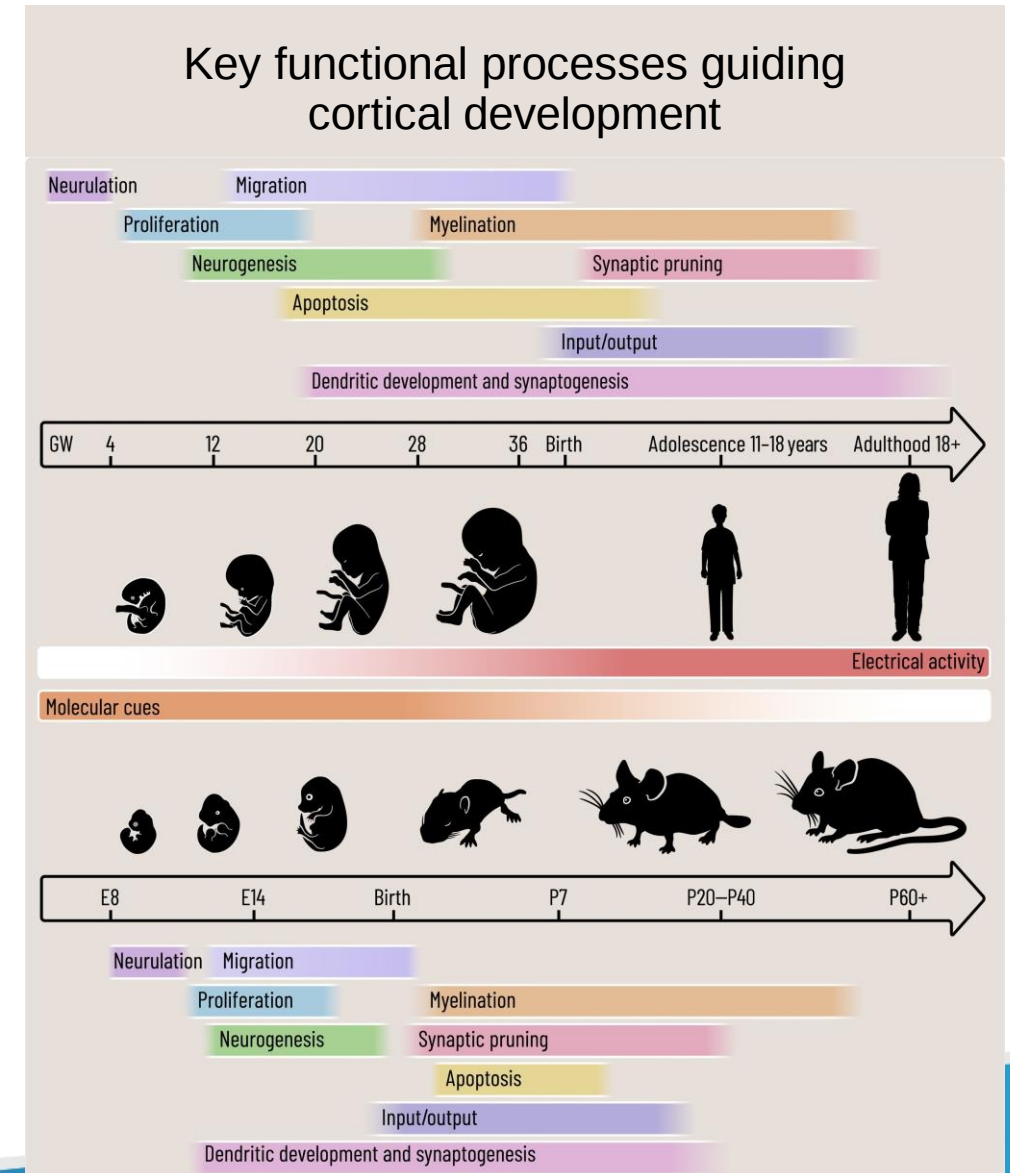
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Webinar Series
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Conflict of Interest Statement

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)

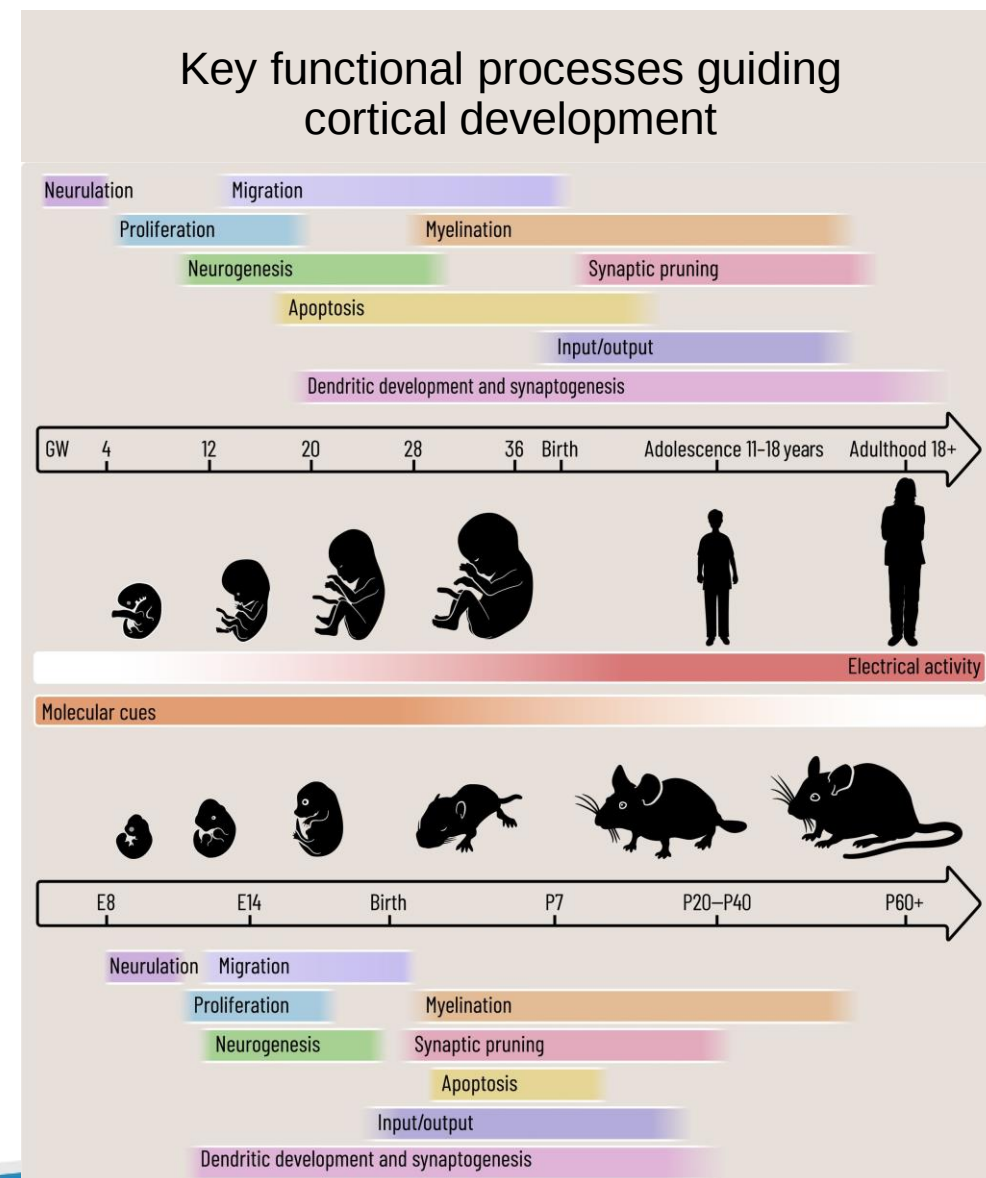
- ❖ Neurodevelopmental disability is the most prevalent chronic medical condition encountered in pediatrics (Zablotsky et al. 2019).
- ❖ Both **genetic and environmental risk factors** have been identified as underlying causes driving this prevalence.
- ❖ DNT NAMs battery: multi-dimensional DNT screening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

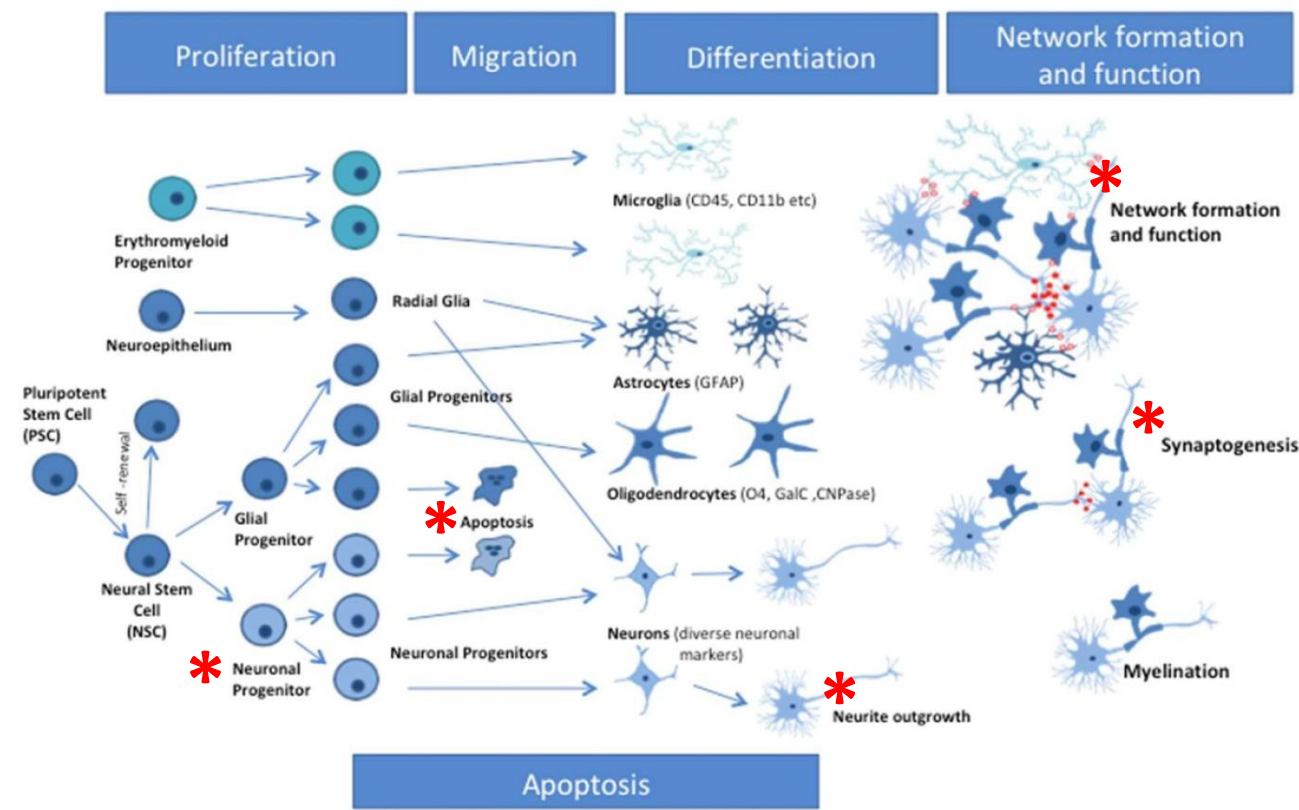
Overview

- 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?



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 NPC1	Harrill et al. (2018) Baumann et al. (2016) and Barenys et al. (2017)
* Apoptosis	UKN1	Balmer et al. (2012)
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

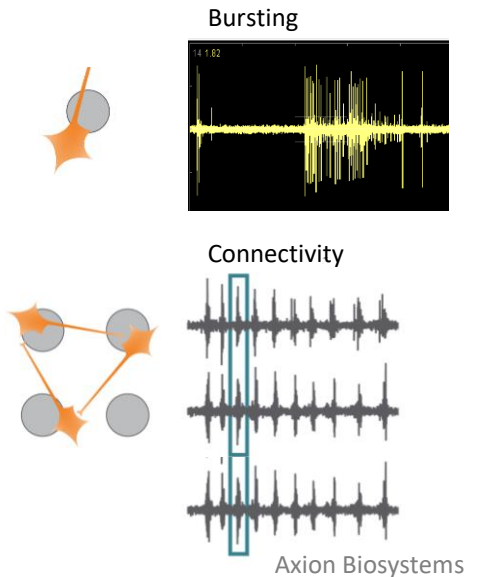
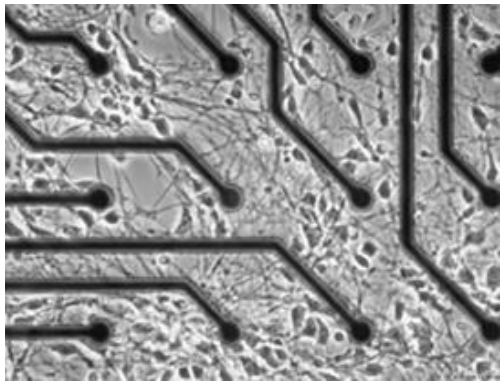
Experimental models in the EPA DNT NAM battery

Microelectrode Array (MEA) Network Formation Assay (NFA)

← 92 chemicals →

High Content Imaging

48-well culture plate
16 electrodes per well



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

96-well culture plate
Immunohistochemistry

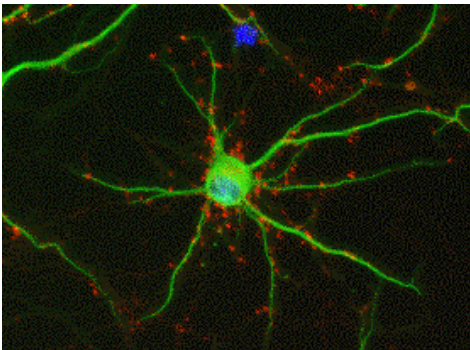
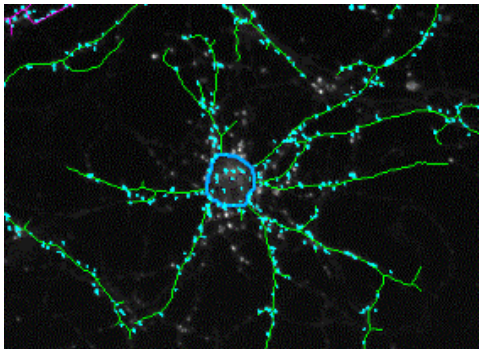


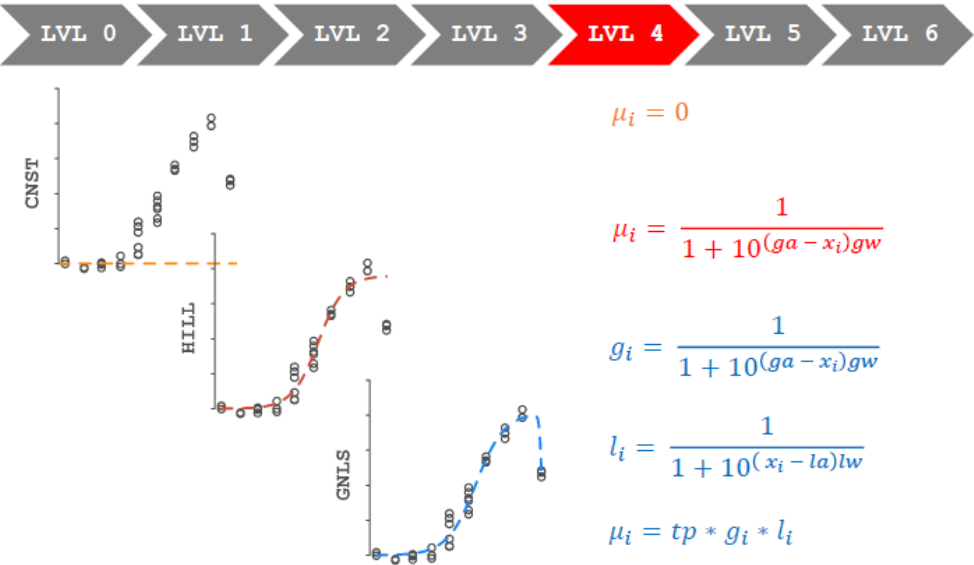
Image Analysis



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

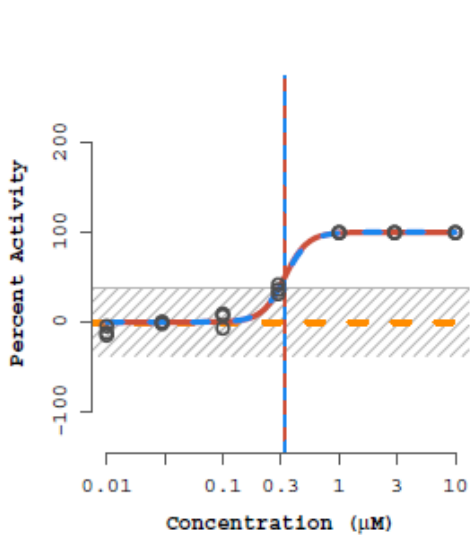
Defining bioactivity using the ToxCast pipeline

Model fitting (constant, hill, gain-loss)



Select winning model and hit-calling

Number of bursting electrodes (down)

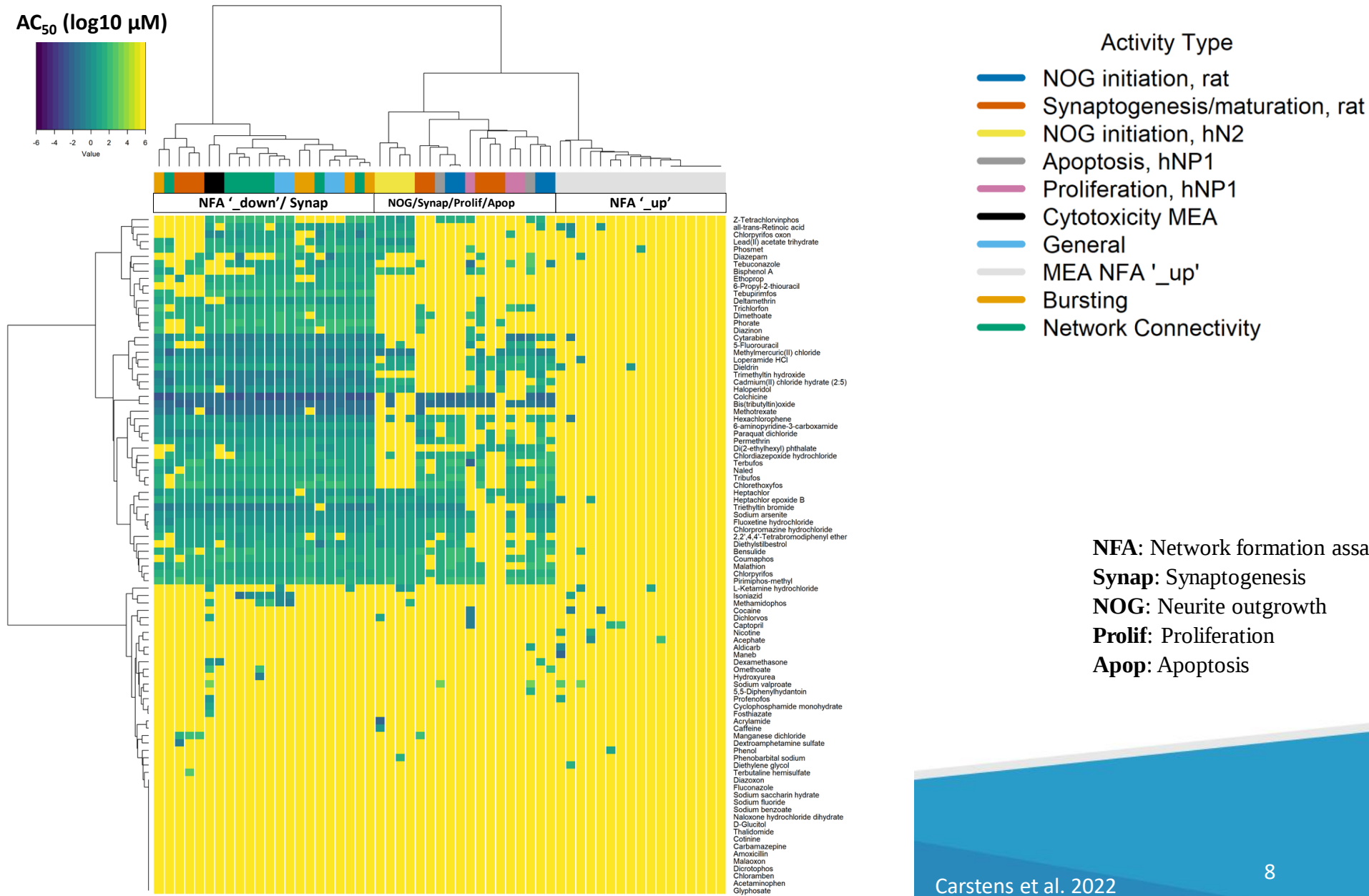


ASSAY:	ABID2500 (CCTE_Shafer_MEA_dev_bursting_electro				
NAME:	Methylmercuric(II) chloride				
CHID:	20813	CASRN:	115-09-3		
SPID(S):	EX000383				
M4ID:	42619839				
HILL MODEL (in red):					
	tp	ga	gw		
val:	100	-0.469	4.43		
sd:	1.28	0.0287	2.18		
GAIN-LOSS MODEL (in blue):					
	tp	ga	gw	la	lw
val:	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	EMAD: 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)

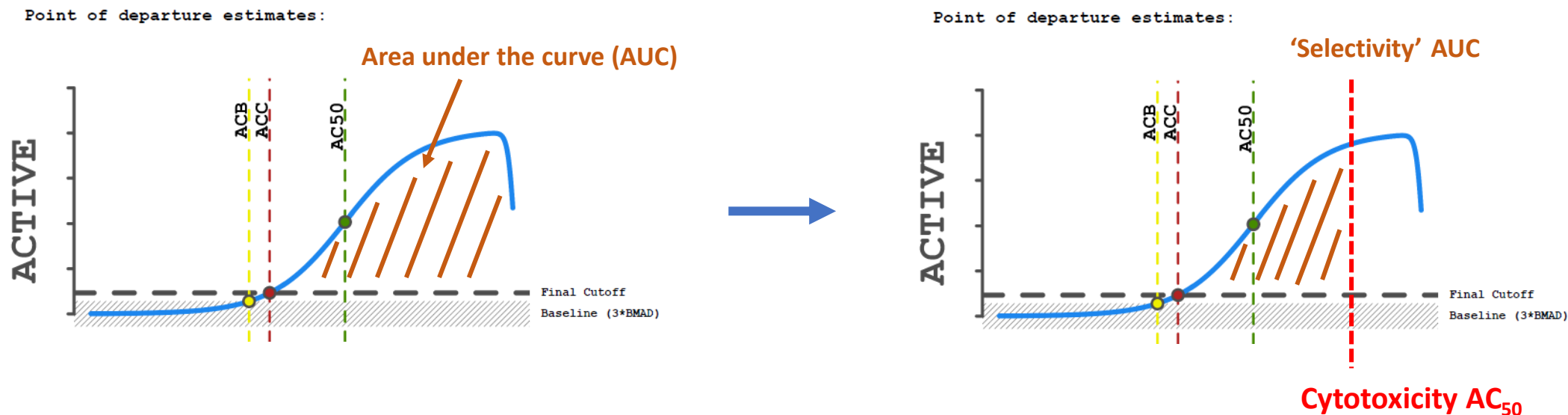
https://cran.r-project.org/web/packages/tcpl/vignettes/Data_processing.html#level-4

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



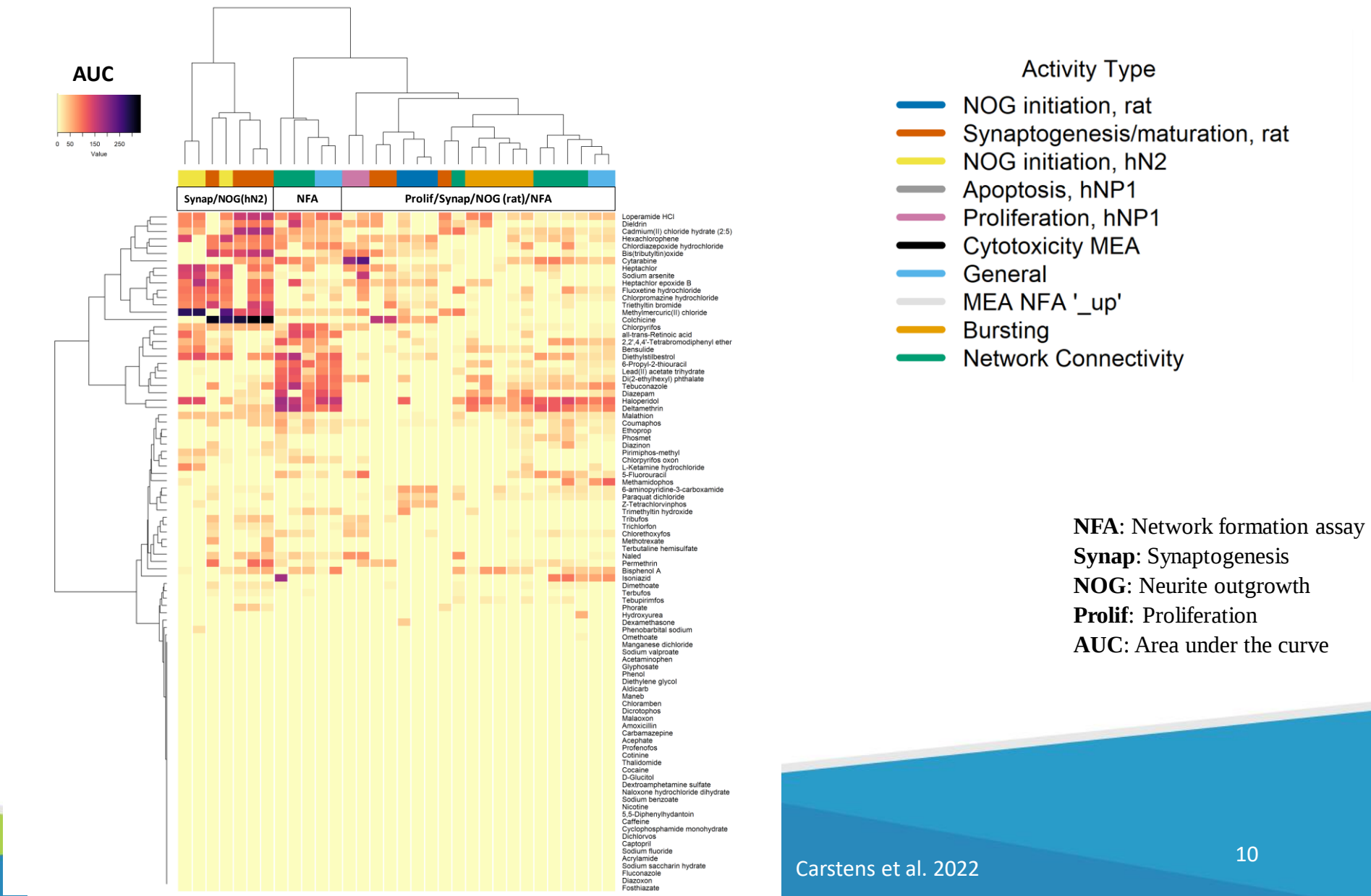
Selectivity: activity at concentrations lower than cytotoxicity

Calculating a *selectivity* metric:



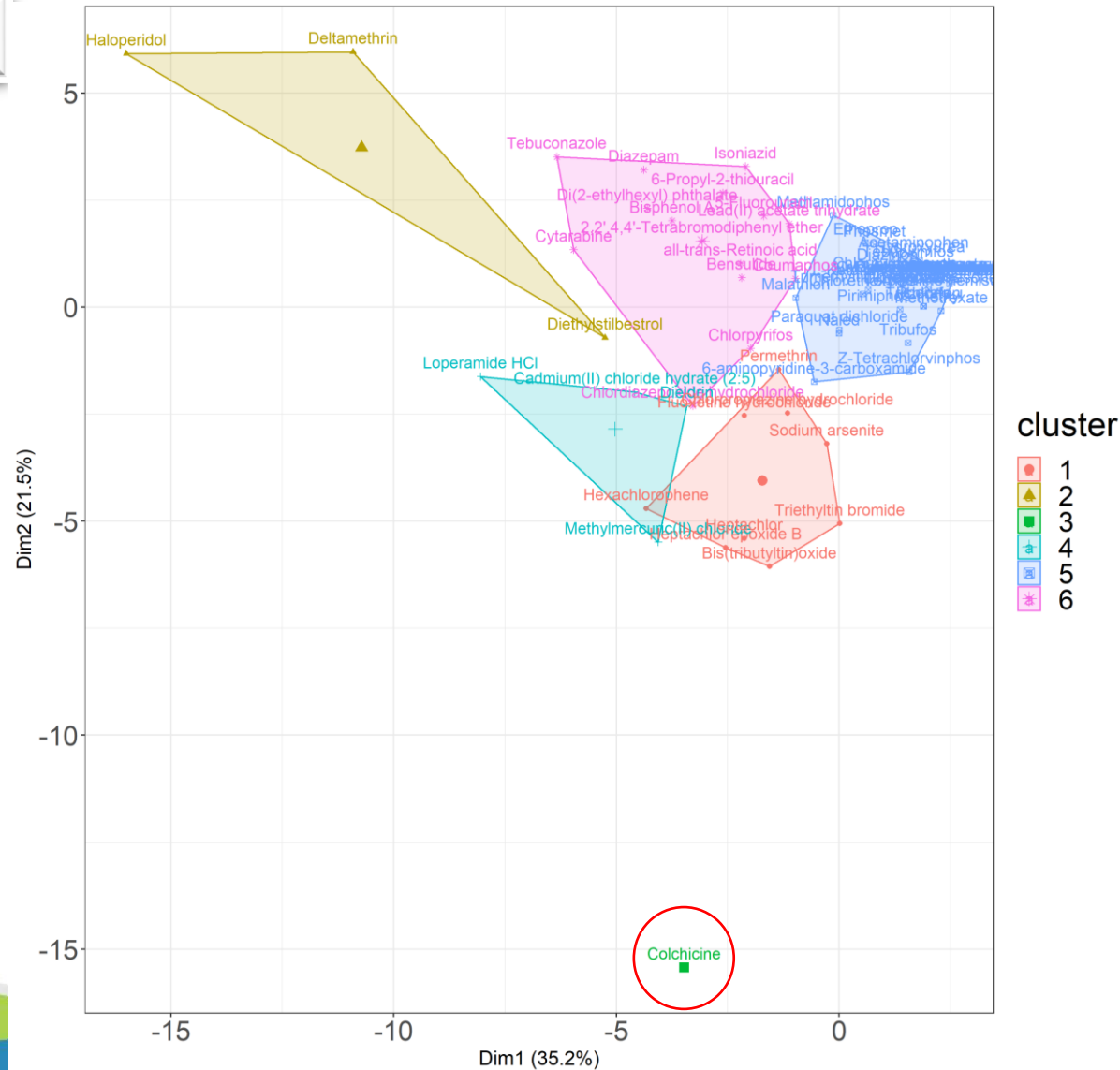
https://cran.r-project.org/web/packages/tcpl/vignettes/Data_processing.html#level-4

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

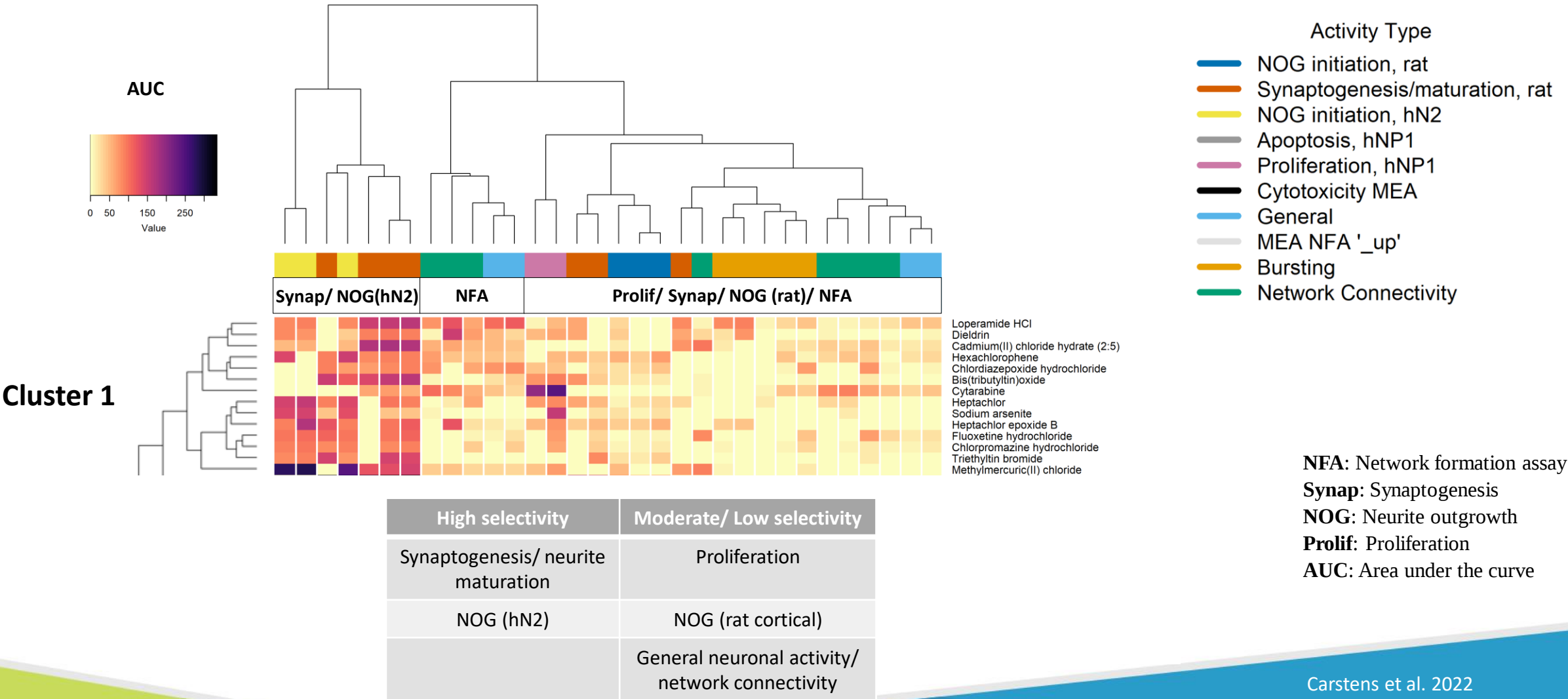


Evaluating ***selectivity*** is informative for identifying patterns of activity.

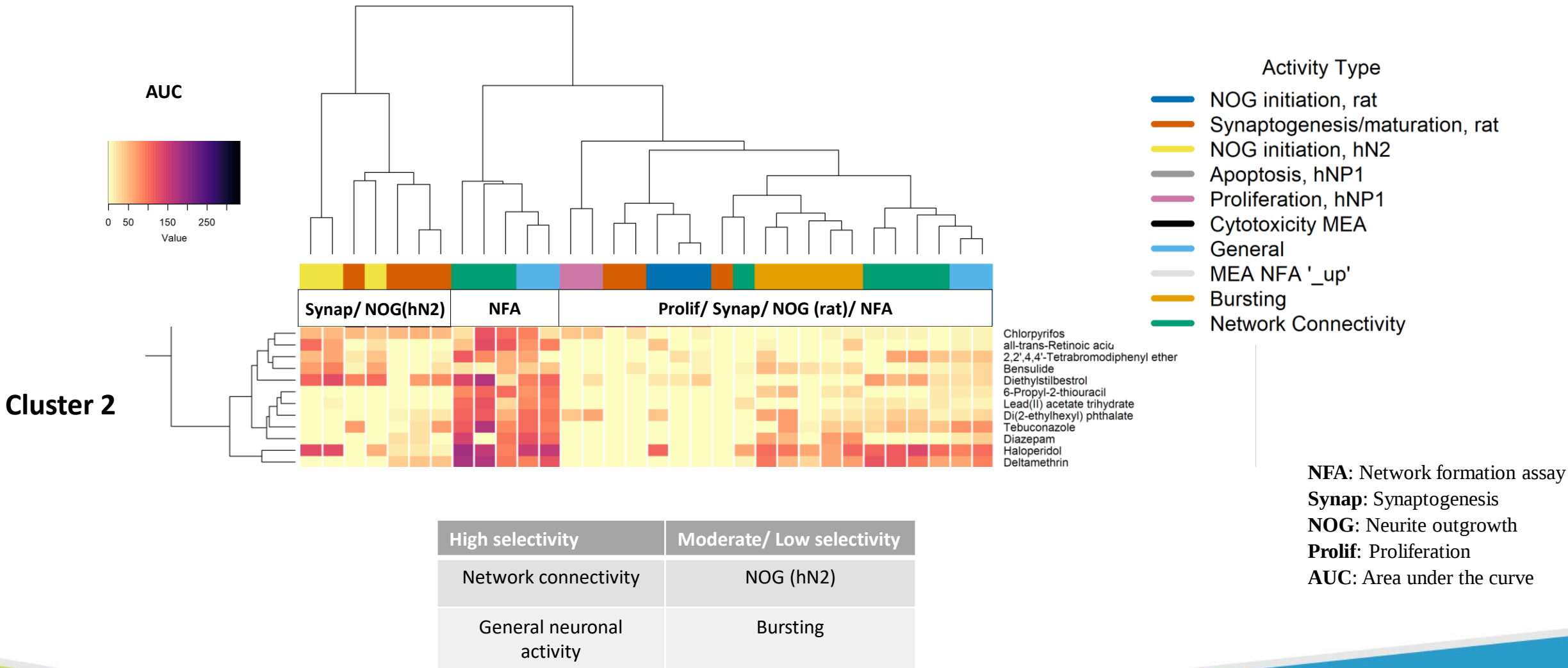
K-means clustering



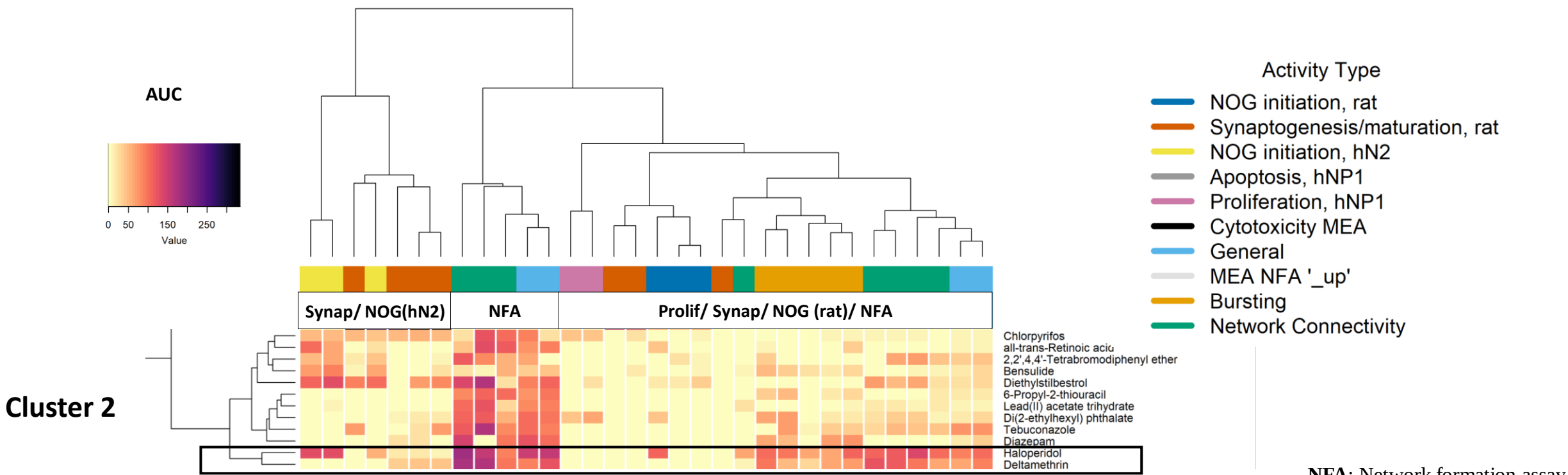
Evaluating *selectivity* is informative for identifying patterns of activity.



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



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



Haloperidol: antipsychotic- Dopamine D₂ receptor antagonist

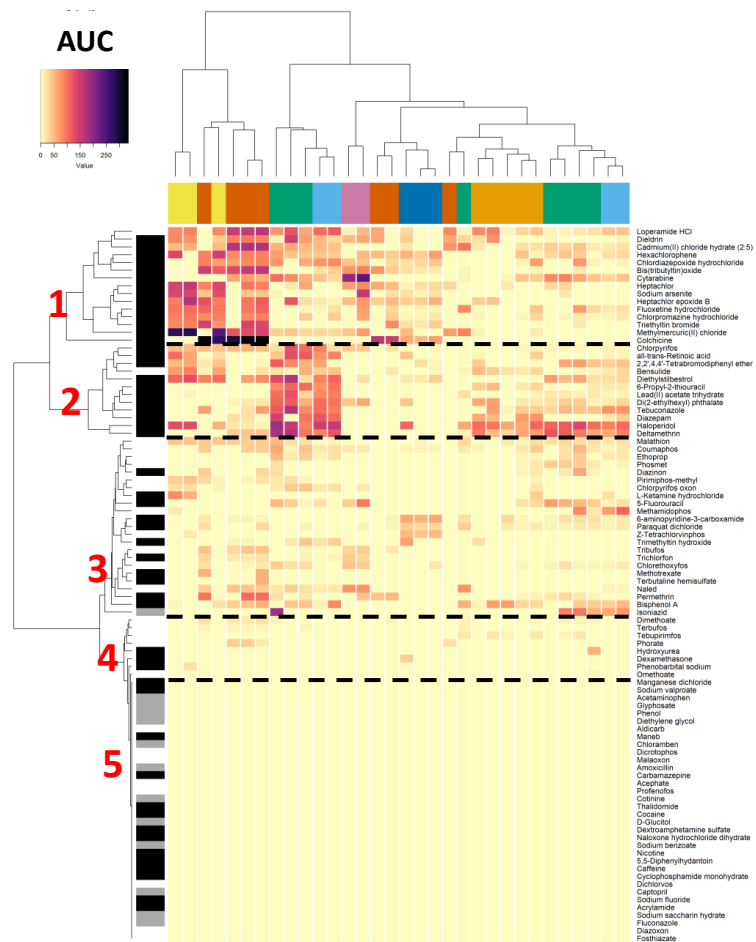
Deltamethrin: pyrethroid insecticide-voltage-gated sodium channels modulators

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

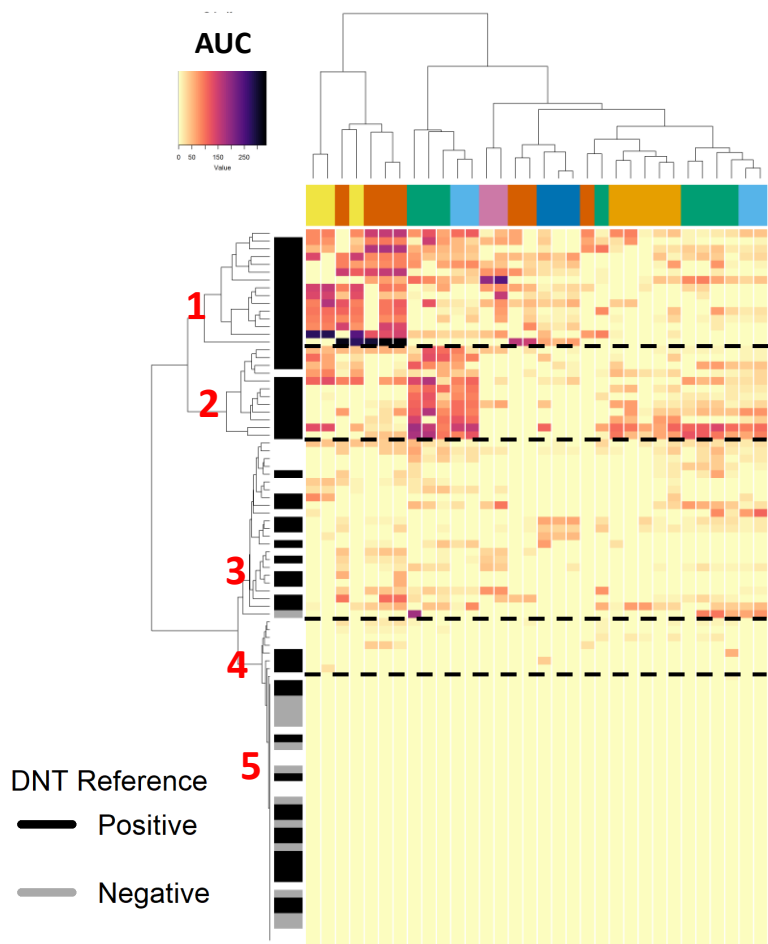
Evaluating *selectivity* is informative for identifying patterns of activity.



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.

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

	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%

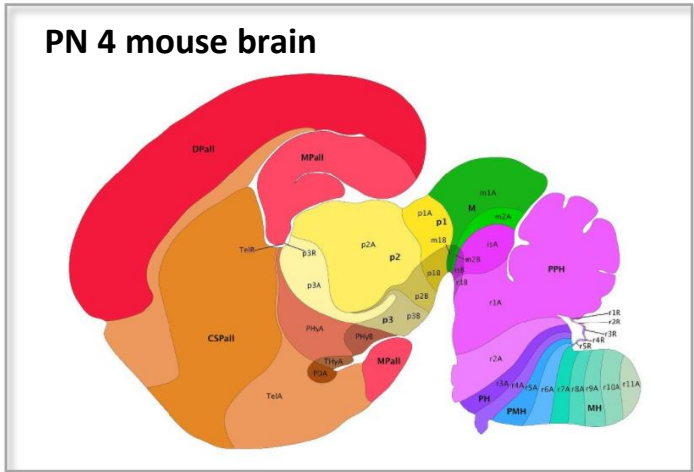
Non-selective
Sensitivity= 93%
Specificity= 69%

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

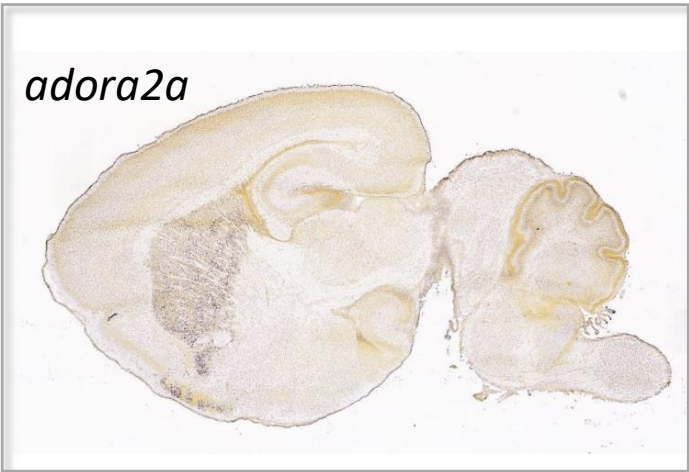
Are we capturing the target mechanism in the DNT NAM battery?

False negative: Caffeine*

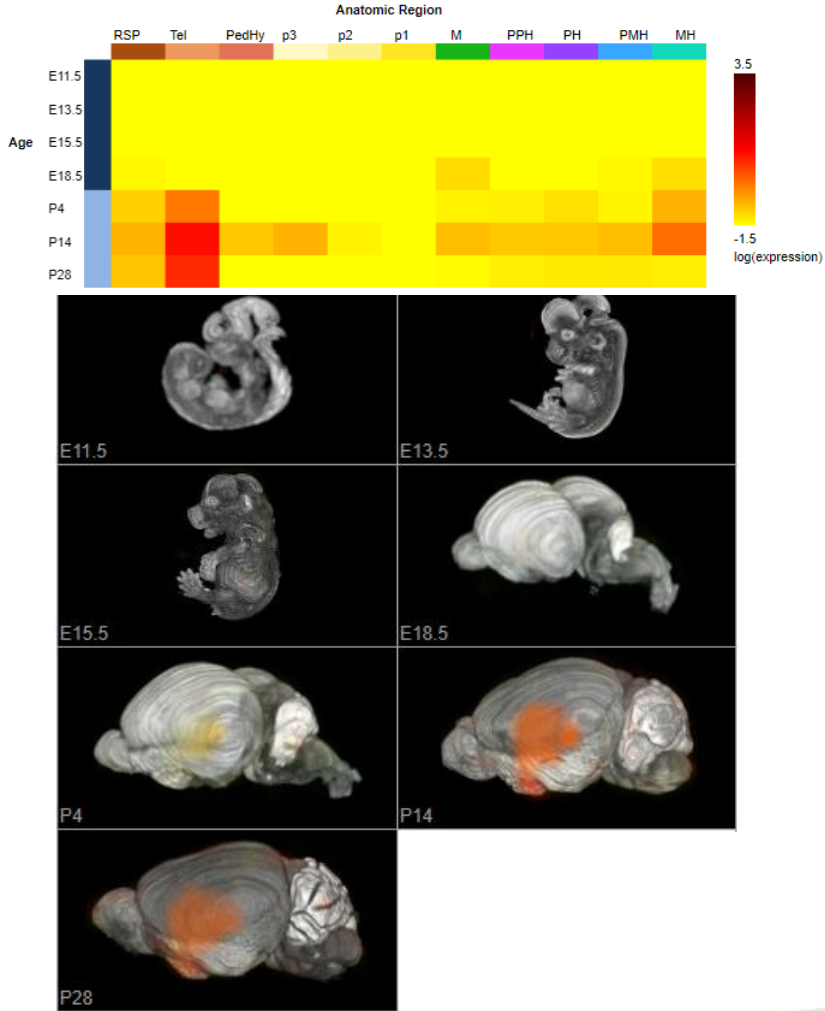
Caffeine targets adenosine receptor (adenosine A2a receptor)



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



In situ hybridization

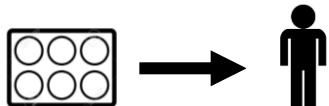


*Reference Mundy et al. (2015) for caffeine as a positive *in vivo* DNT evaluation chemical.

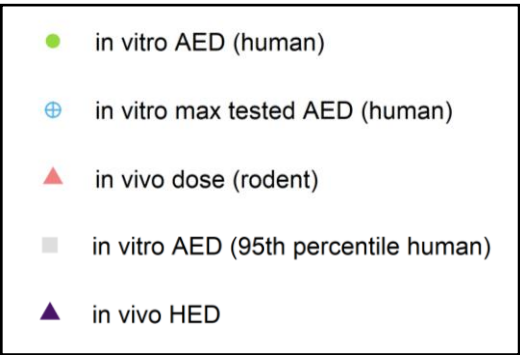
In vitro to in vivo extrapolation (IVIVE) using high-throughput toxicokinetic (HTTK) modeling

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

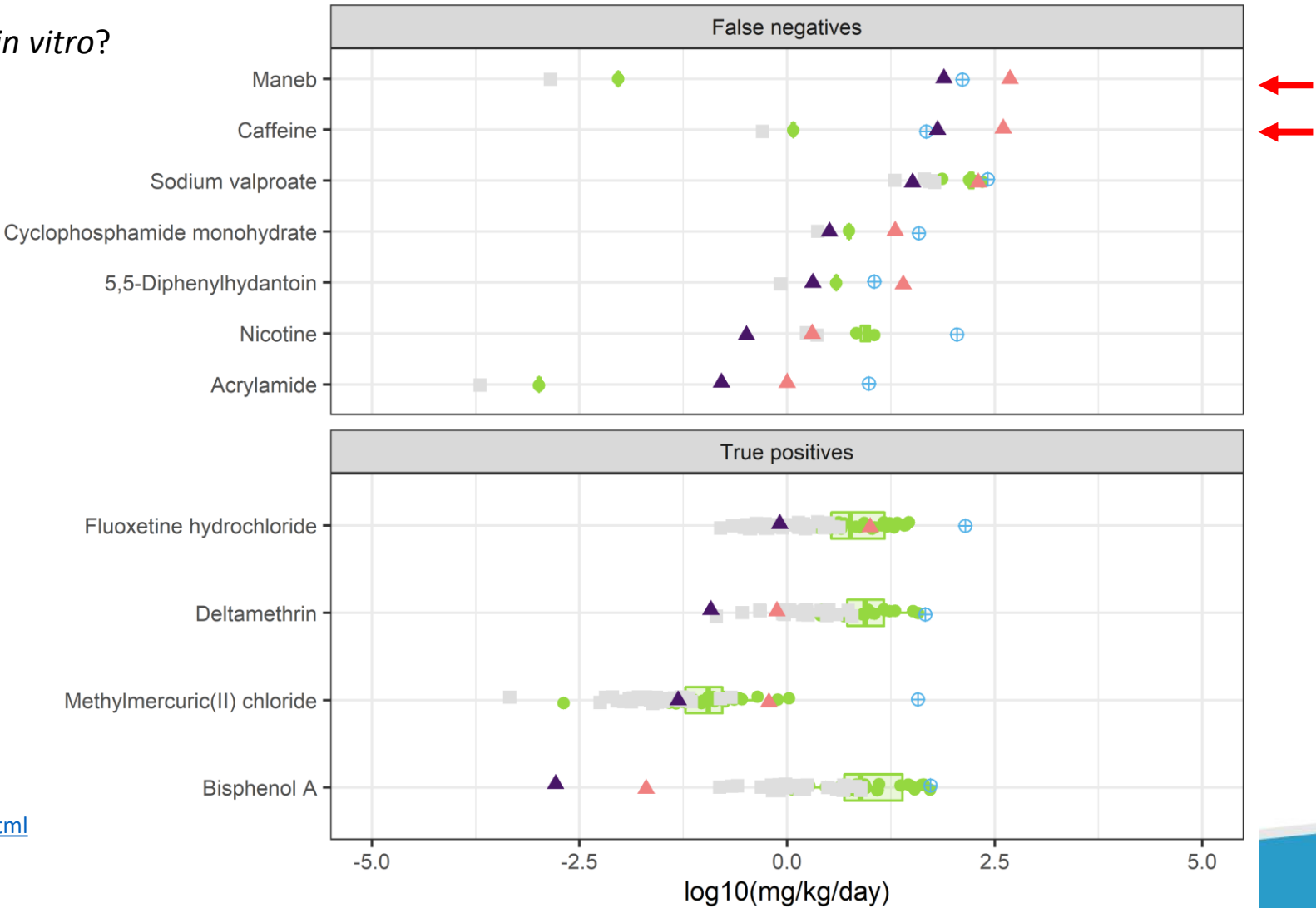
AED: administered equivalent dose



HED: human equivalent dose



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



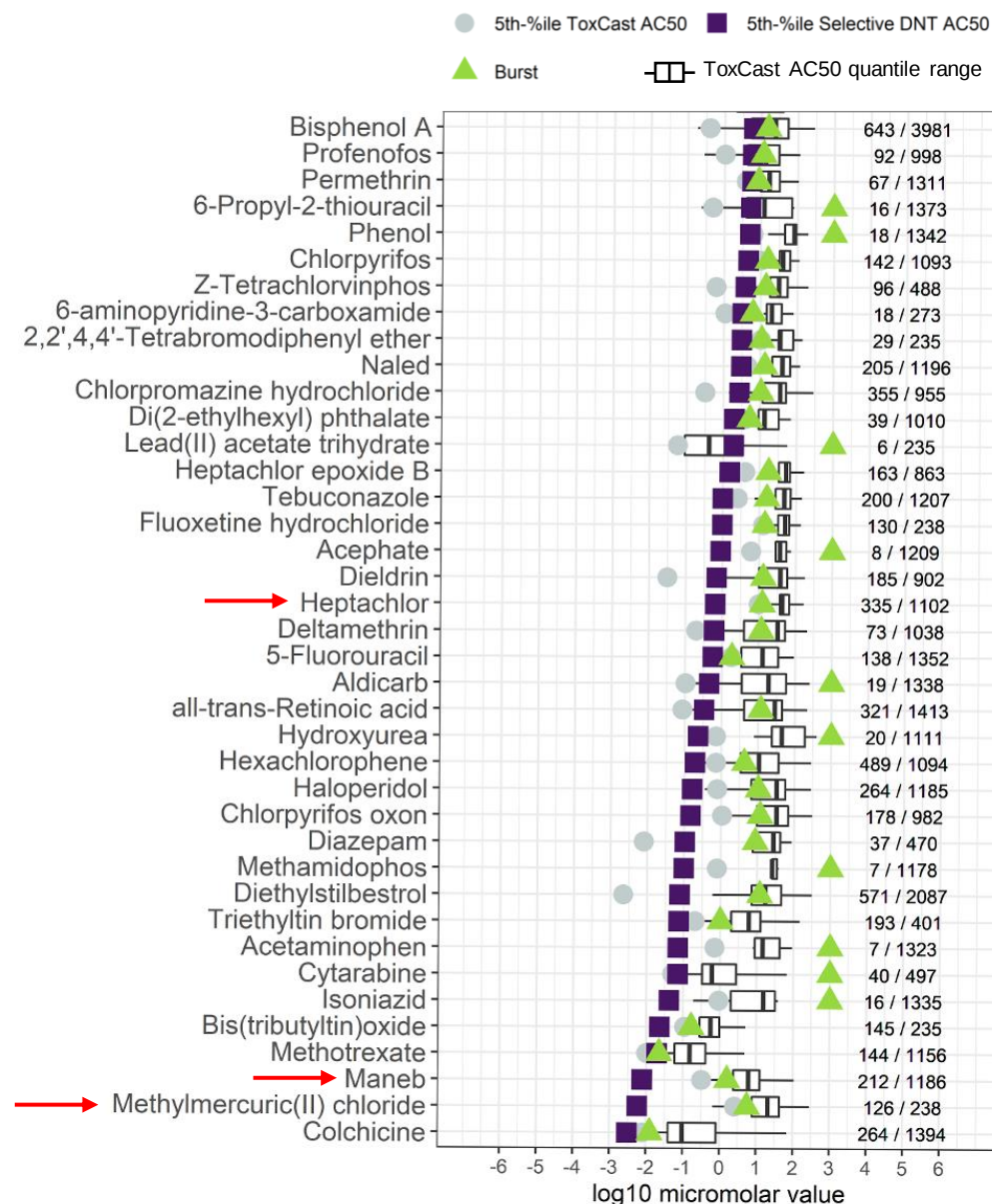
Comparison of *selective* DNT NAM activity to ToxCast/Tox21 database

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?

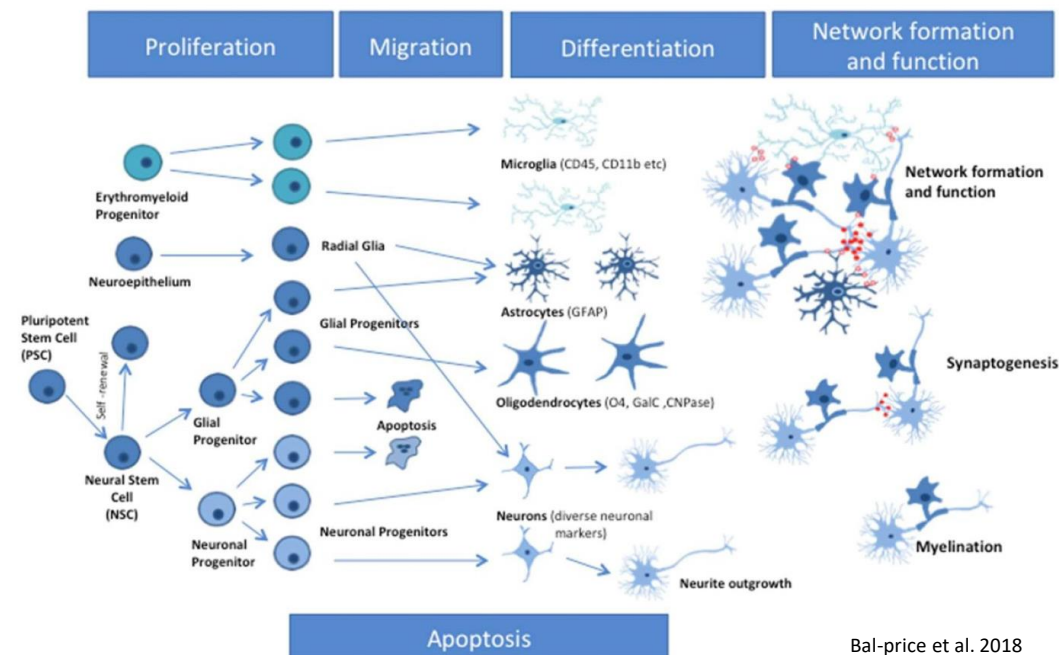
- Selective data is more informative in identifying differential patterns of functional bioactivity than non-selective data.
- Selective activity clusters do not necessarily appear to be explained by mode-of-action.

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.

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.



Bal-price et al. 2018



Questions?

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Assay data:

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