DNT Journal Club 06/30/2021

Computational approaches to evaluate *in vitro* New Approach Methodologies (NAMs) for Developmental Neurotoxicity (DNT)

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Background on developmental neurotoxicity (DNT) screening

DNT: any adverse outcome of exposure to a toxic substance on the normal development of the nervous system structure and/or function

DNT Guideline Study limitations:

- resource intensive (time/ cost/ animals)
- only ~150 compounds have DNT Guideline Studies & not often used for point-of-departure (25%) values for risk assessment
- mechanism: apical endpoints with little information on underlying biological process

Individual DNT- New Approach Methodologies (NAMs) *in vitro* assay limitations:

-some compounds may disrupt key cellular events at different states of development
-some compounds may disrupt distinct cellular events throughout neurodevelopment
-some neural cell-types may be differentially sensitive to perturbation
-no single *in vitro* screening assay can recapitulate all critical cellular events of neurodevelopment

Battery of DNT-NAMs:

- multi-dimensional high-throughput DNT screening assays; rapid data generation, cost-effective, limit animal-use
- cover complex biological space: temporal, cell-type, species, different cellular events of neurodevelopment

Background on DNT-NAM battery

Two DNT-NAM technologies:

1) Microelectrode array (MEA) network formation assay (NFA)
2) High-content imaging (HCI)

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 (rat cortical)	Brown *et al.* (2016) and Frank *et al.* (2018)

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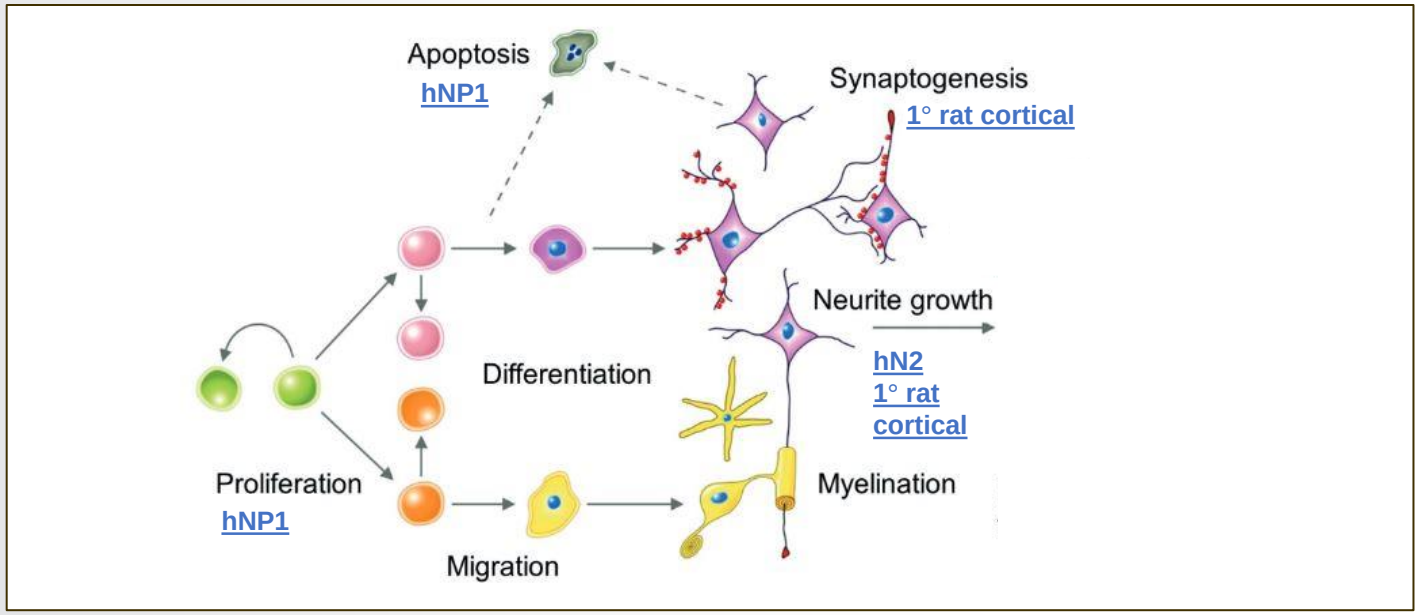

Early-stage Developement → **Late-stage development**

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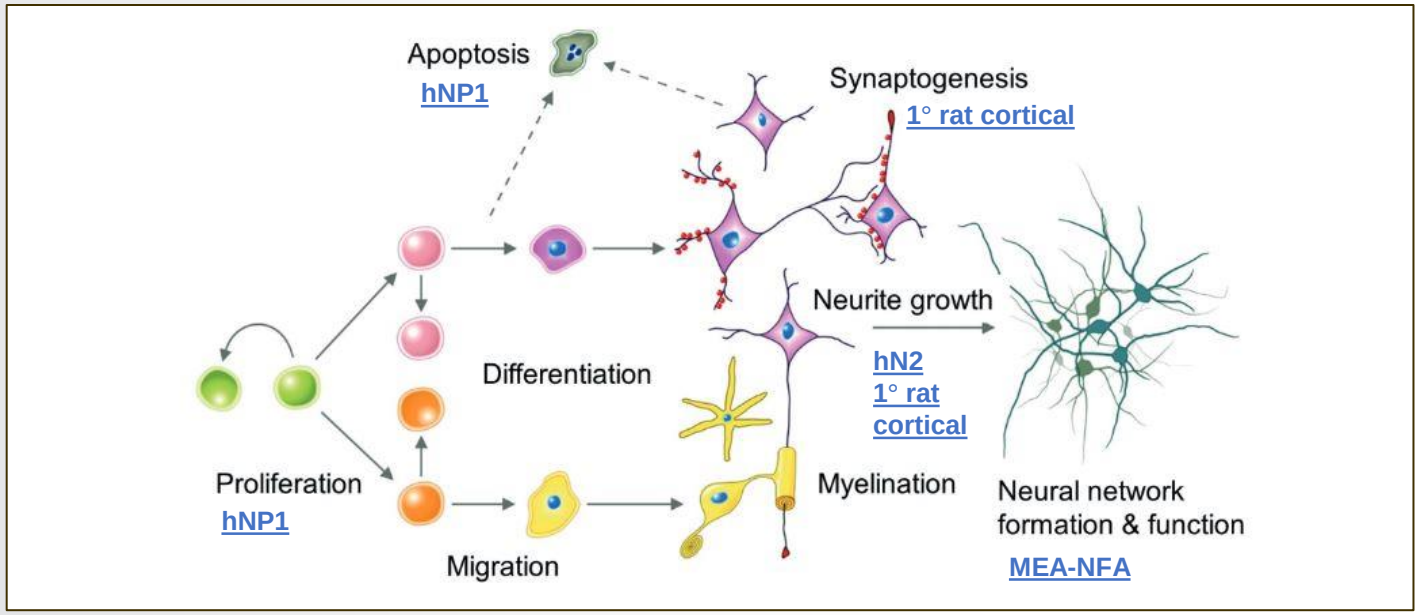

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Aim:

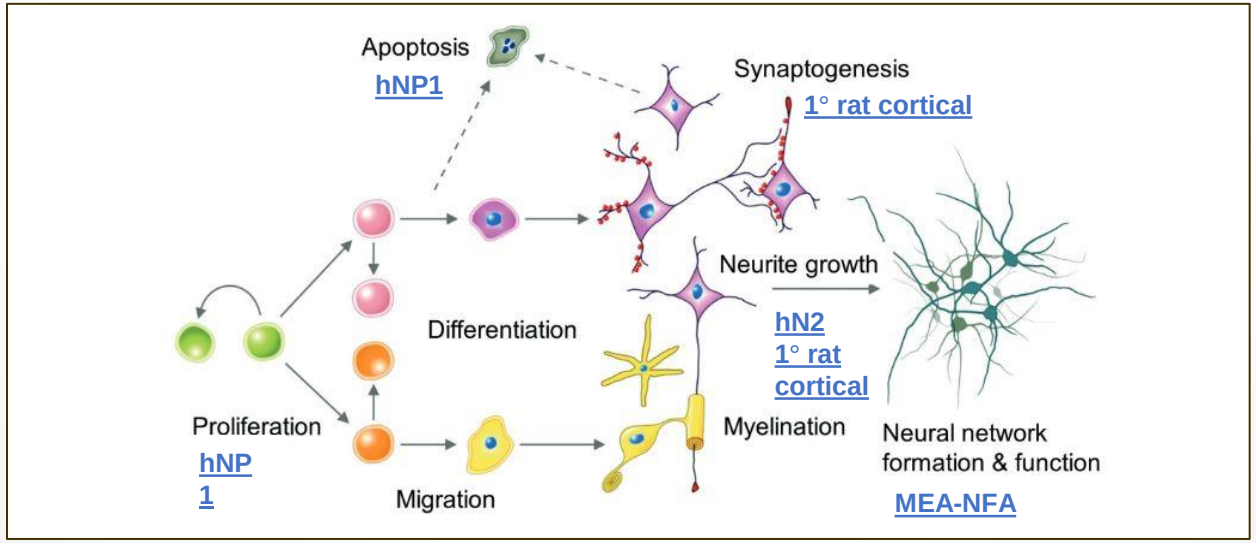
Develop a battery of DNT-NAMs for fit-for-purpose evaluation of DNT.

Questions:

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

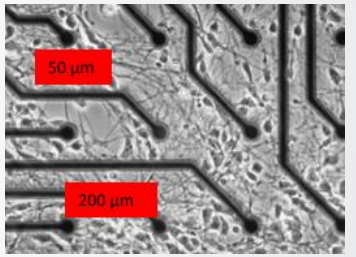
2) Does the DNT-NAM battery classify *in vivo* DNT reference chemicals?

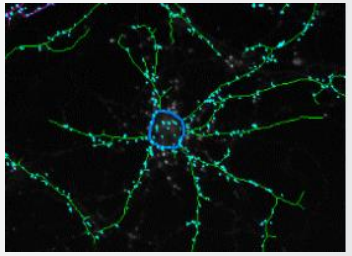
3) Can we use the DNT-NAM battery to identify the most sensitive endpoints?



92 Chemicals tested in NFA and HCI assays

Assay technology name	Dataset in invitrodb	Chemicals tested in both technologies	Cell culture	Assay/ key cellular events	Number of endpoints measured
NFA: network formation assay	CCTE_Shafer_MEA_dev_	92 (28 repeats)	Primary rat cortical neurons (DIV 5, 7, 9, 12)	Microelectrode array (MEA); Decreasing neuronal activity (network/ general/ bursting)	17
				Increasing neuronal activity	17
				Cytotoxicity	2
HCI: high-content imaging assays	MUNDY_HCI_	92	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

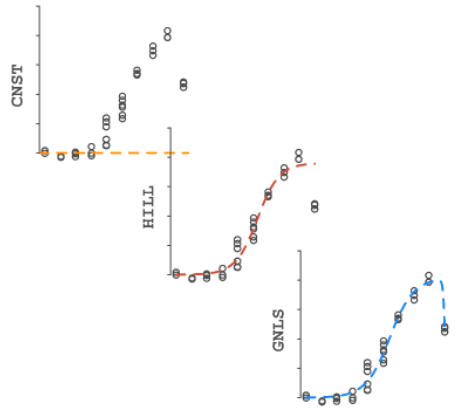




How do we define an ‘active’ DNT compound?

1) Model fitting (constant, hill, gain-loss)

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



$$\mu_i = 0$$

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

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

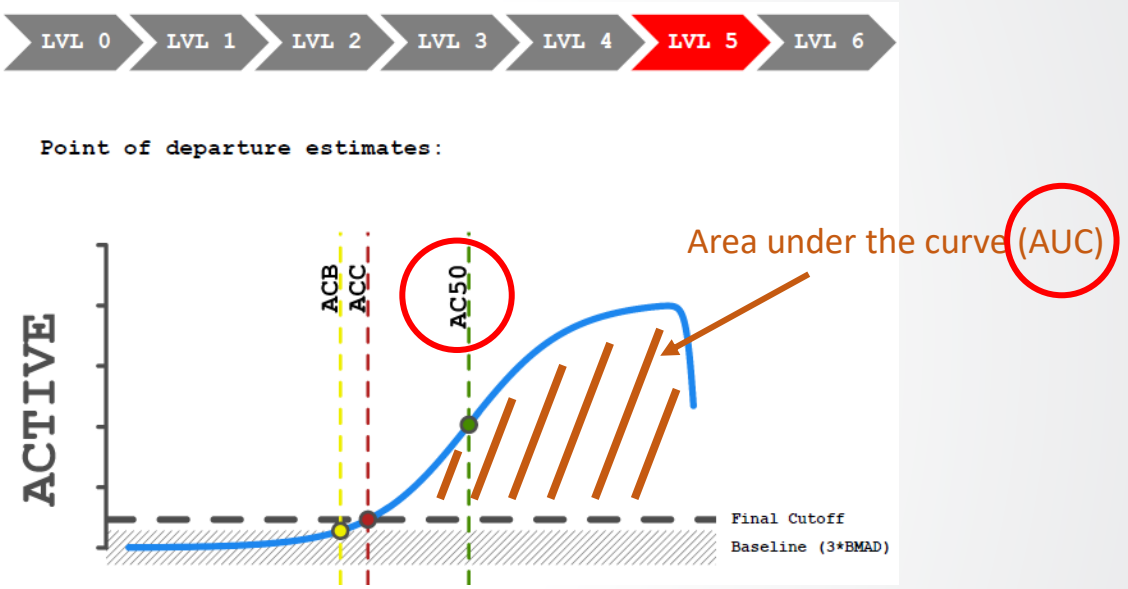
$$l_i = \frac{1}{1 + 10^{(x_i - la)lw}}$$

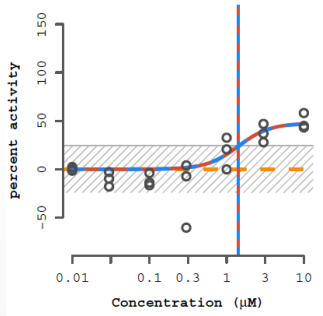
$$\mu_i = tp * g_i * l_i$$

Where u_i and x_i are the modeled response and log concentration at the i^{th} observation, respectively, ga is the gain log(AC50), gw is the gain Hill coefficient, la is the loss log(AC50), and la is the loss Hill coefficent.

2) Select winning model and hit-calling

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





ASSAY: AEID2529 (CCTE_Shafer_MEA_dev_LDH_dn)

NAME: Profenofos
CHID: 32464 CASRN: 41198-08-7
SPID(S): TT0000177A01
M4ID: 41366637

HILL MODEL (in red):

	tp	ga	gw
val:	47.5	0.15	2.13
sd:	7.7	0.2	0.991

GAIN-LOSS MODEL (in blue):

	tp	ga	gw	la	lw
val:	47.5	0.15	2.13	2.83	4.12
sd:	7.7	0.2	0.991	1390	3140

	CNST	HILL	GNLS
AIC:	203.9	177.04	181.04
PROB:	0	0.88	0.12
RMSE:	29.03	16.6	16.6

MAX_MEAN: 48.7 MAX_MED: 44.8 BMAD: 8.03

COFF: 24.1 HIT-CALL: 1 FITC: 41 ACTP: 1

FLAGS: 17

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

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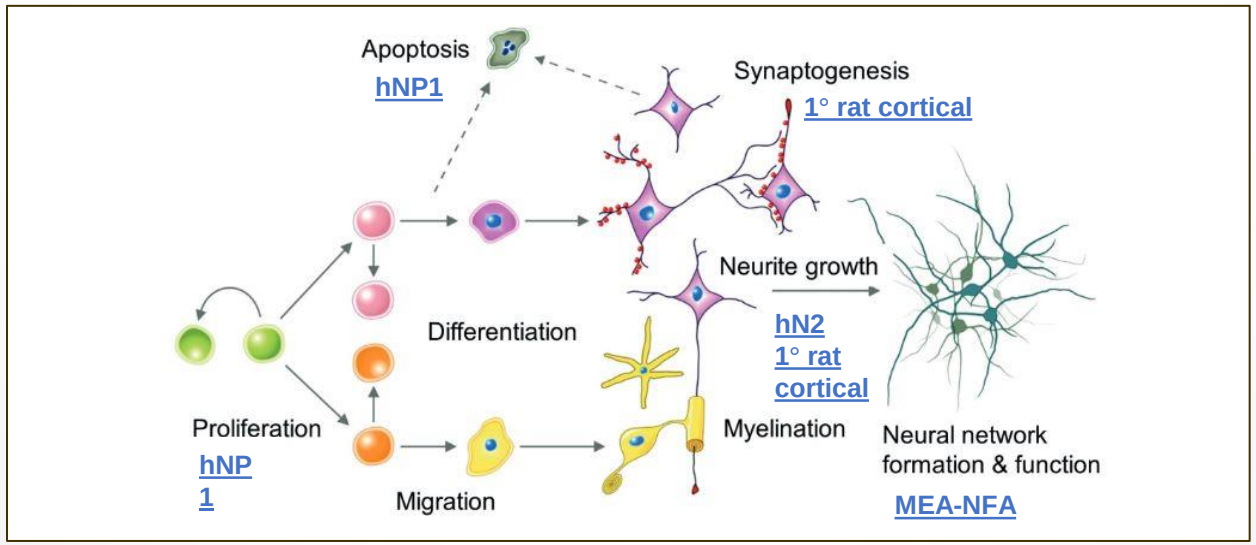
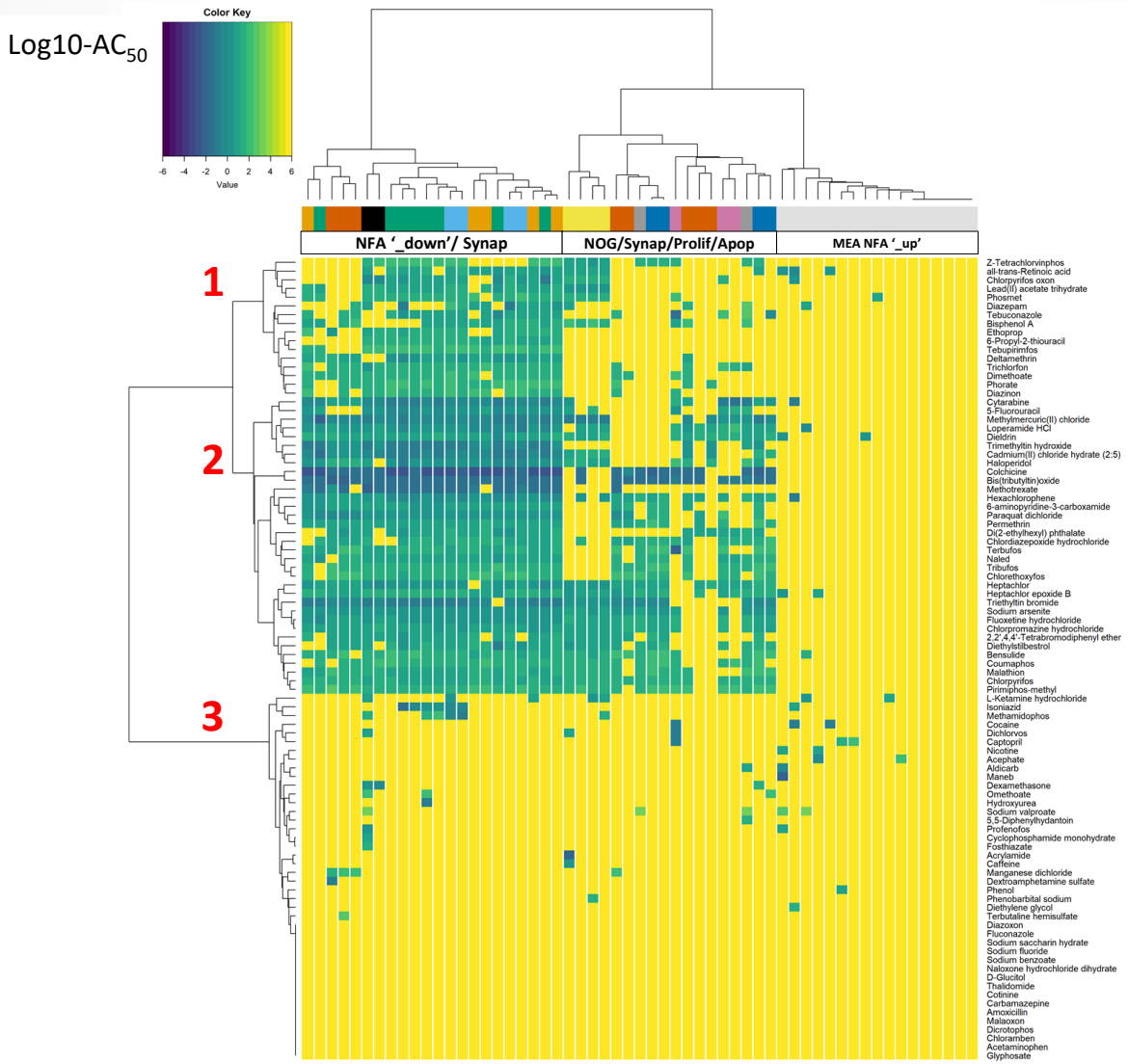


How does the battery collectively inform DNT-relevant bioactivity?



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

What is the relationship between the 57 DNT-NAM endpoints?

NOG (rat cortical)

Synaptogenesis/ maturation (rat cortical)

NOG (human hN2)

Proliferation (hNP1)

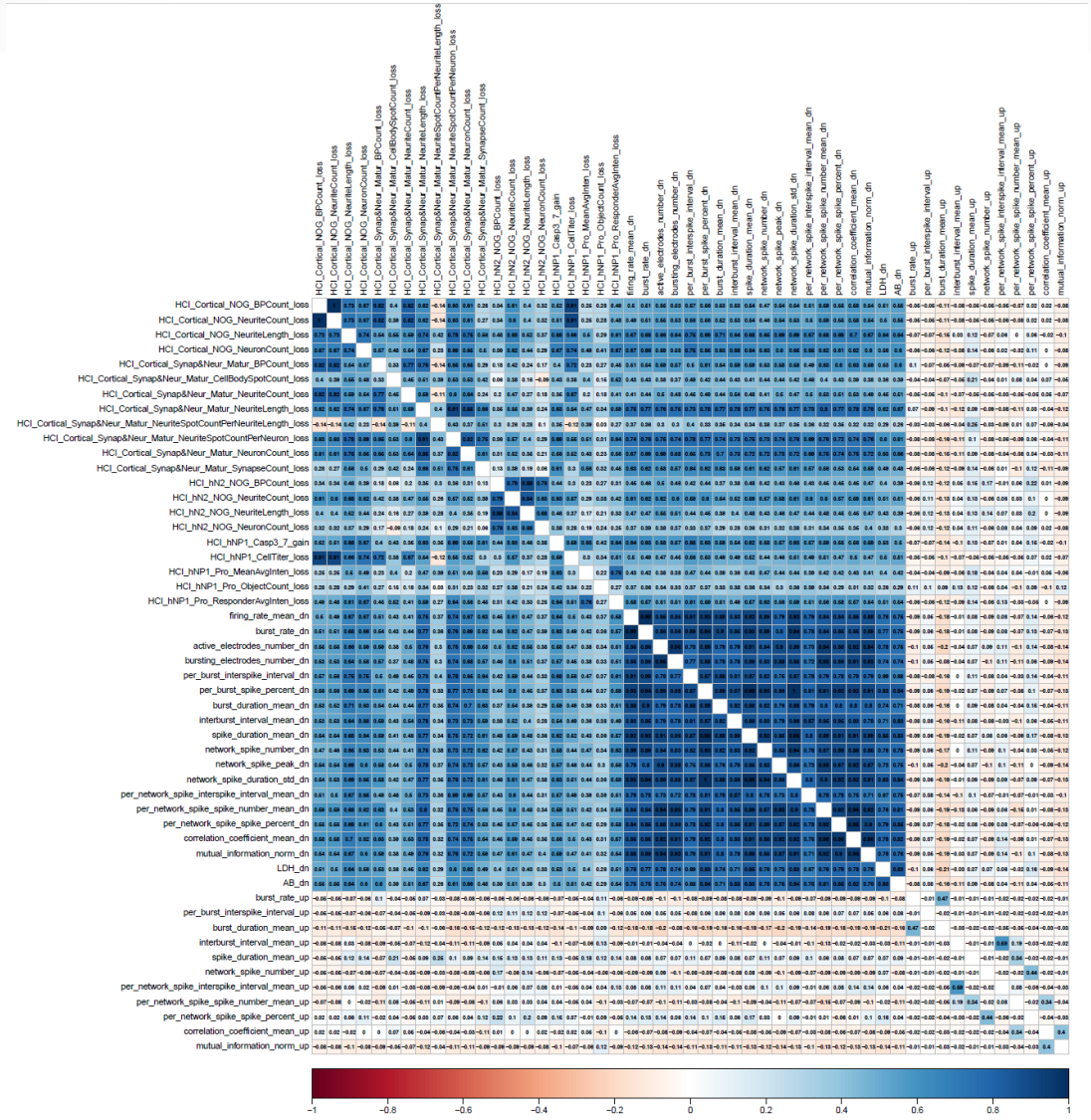
General activity

Bursting

Network activity

MEA cytotoxicity

Increasing NFA activity

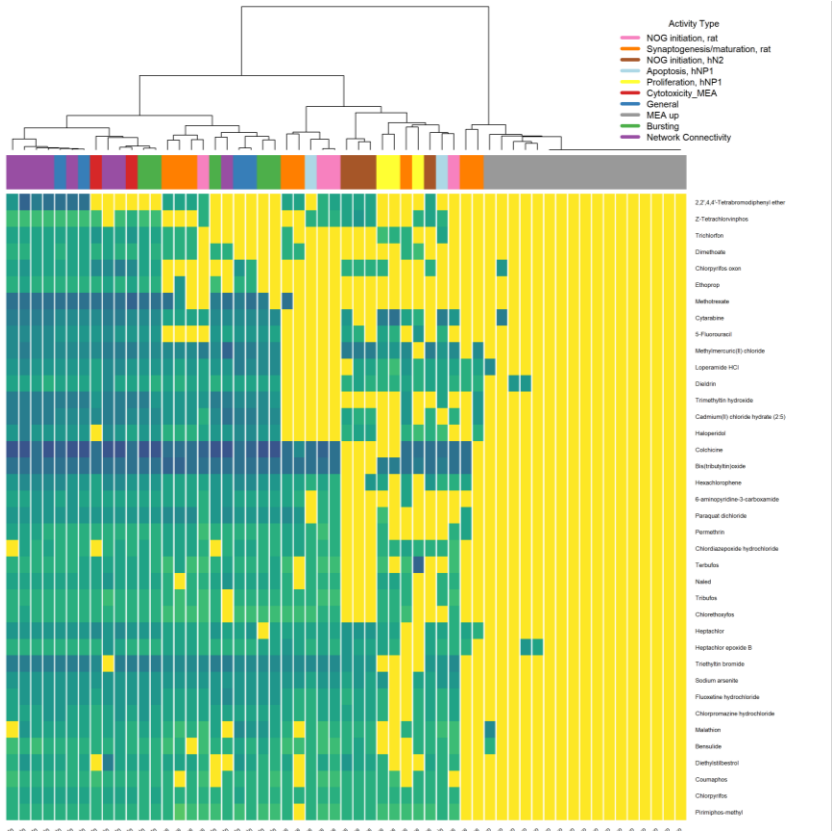


MEA: microelectrode array
NOG: Neurite outgrowth

Do cytotoxic effects account for ‘active’ compounds in the DNT battery?

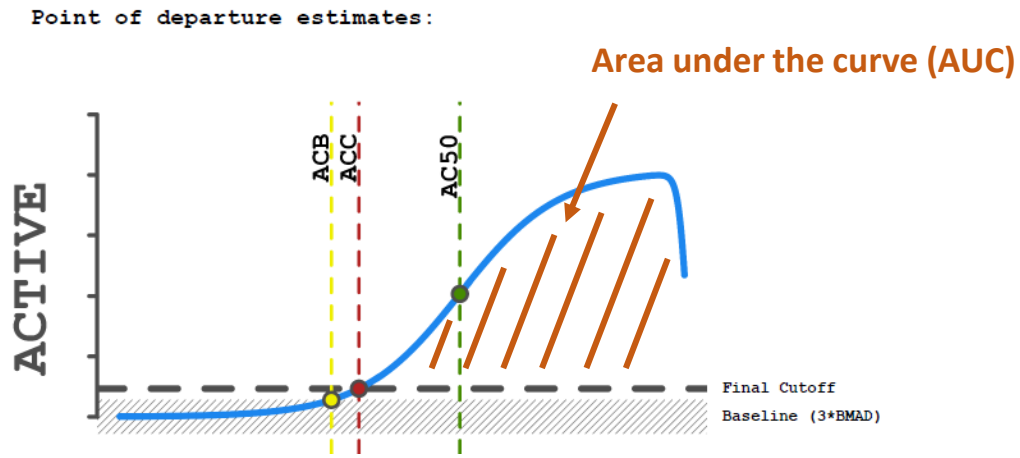
Activity in 8 cytotoxicity endpoints

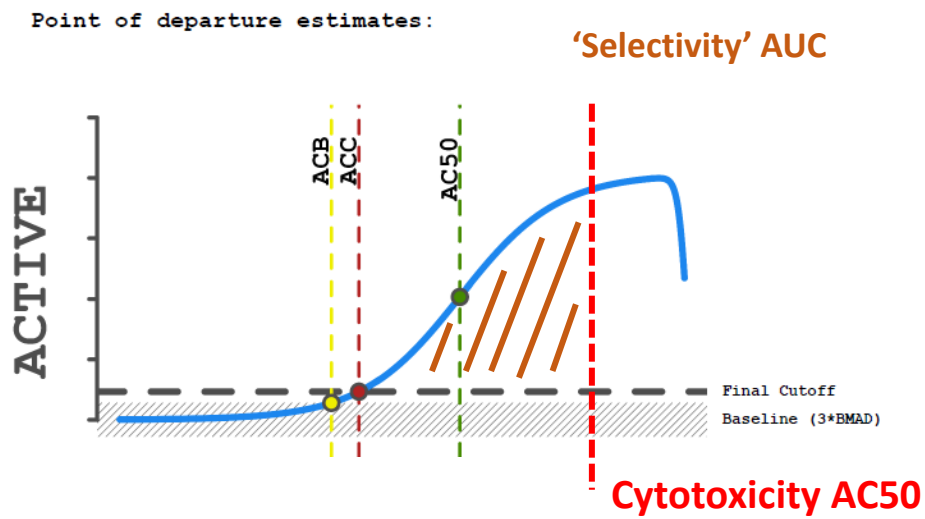
Activity in the DNT NAM battery



Calculating a ‘selectivity’ metric

Selectivity: activity at concentrations lower than cytotoxicity.





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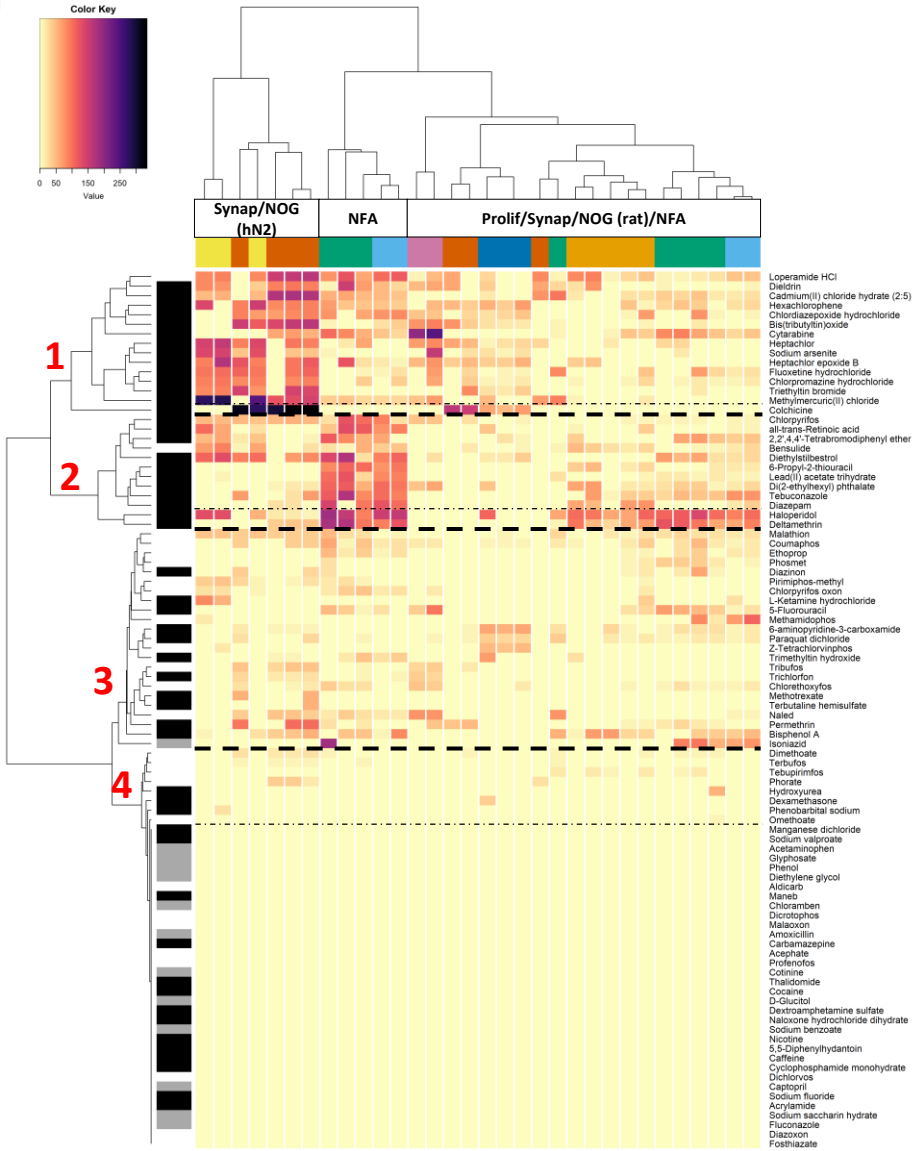
Is evaluating 'selectivity' more informative for identifying patterns of DNT activity?



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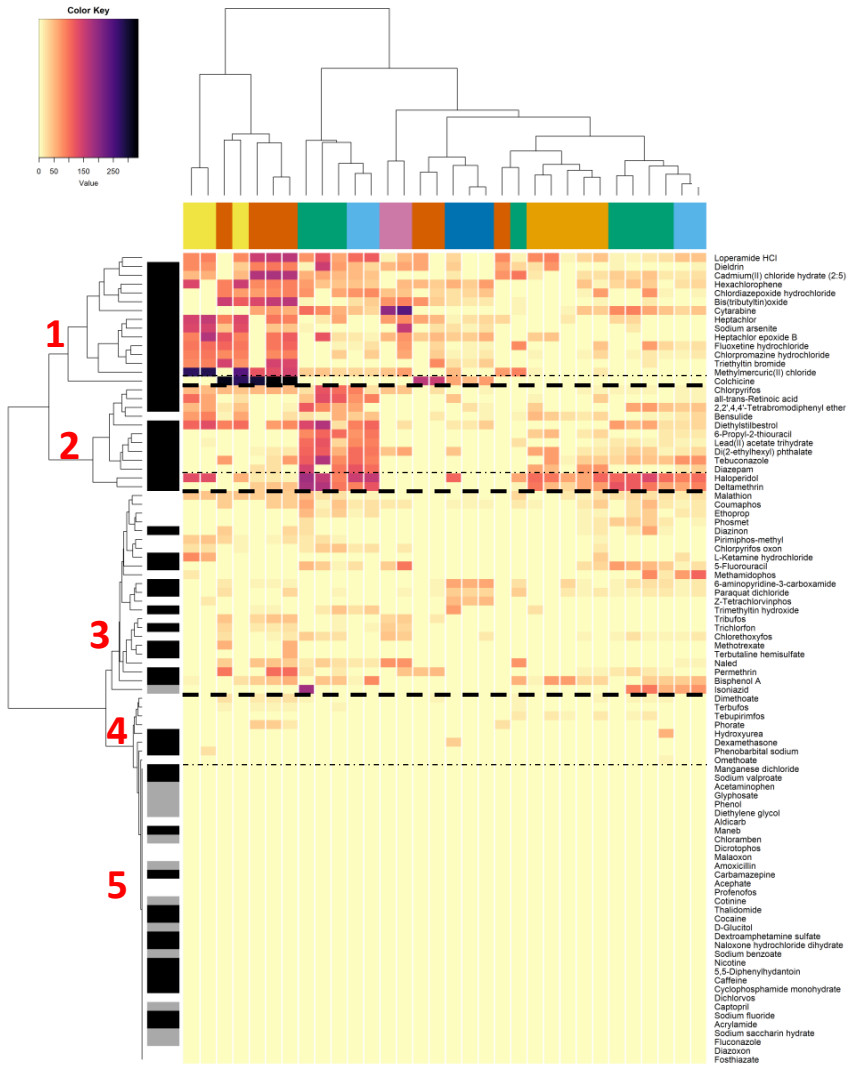
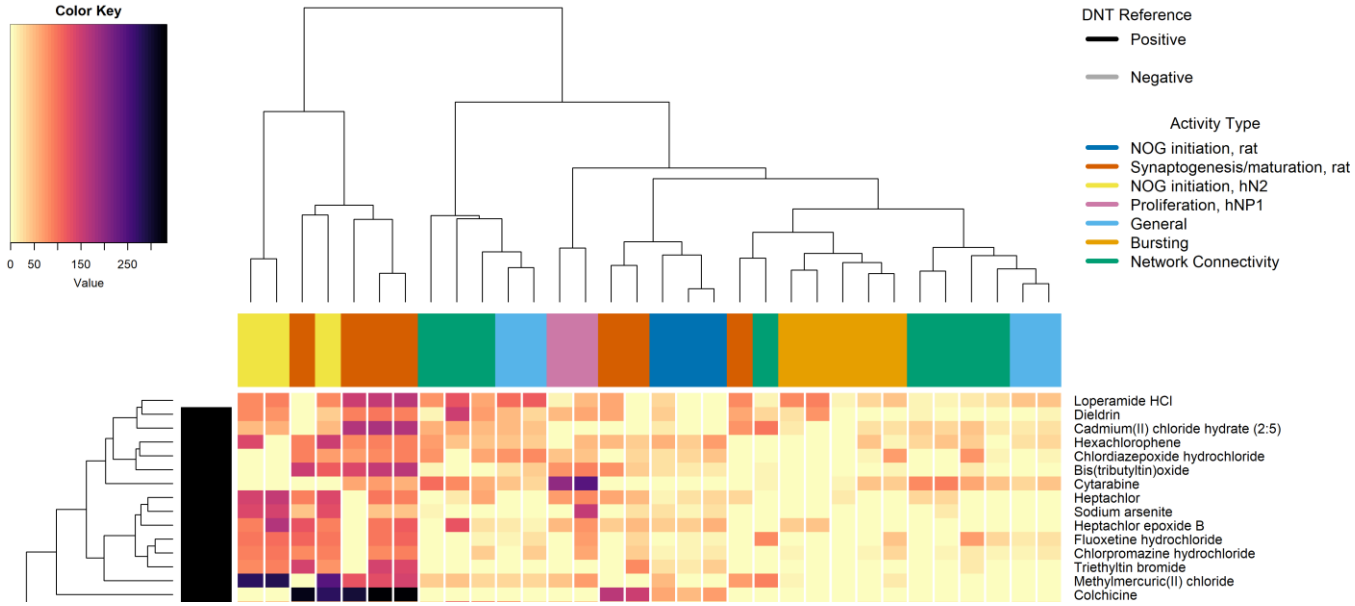


Cluster 1



High selectivity	Moderate/ Low selectivity
Synaptogenesis/ neurite maturation: -neurite length loss -number of puncta per neurite -synapse count loss	Proliferation/ NOG (rat cortical)/ network formation
NOG (hN2): -neurite count loss -neurite length loss -branch point count loss	

NOG: Neurite outgrowth

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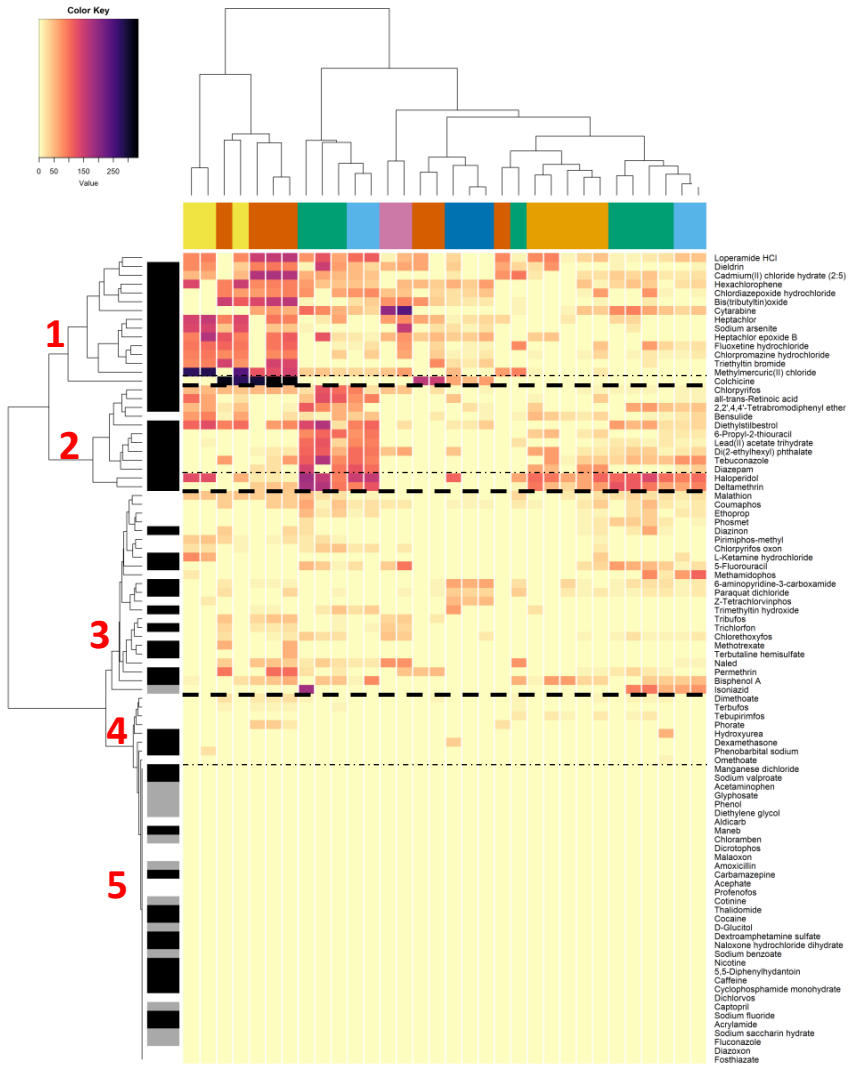
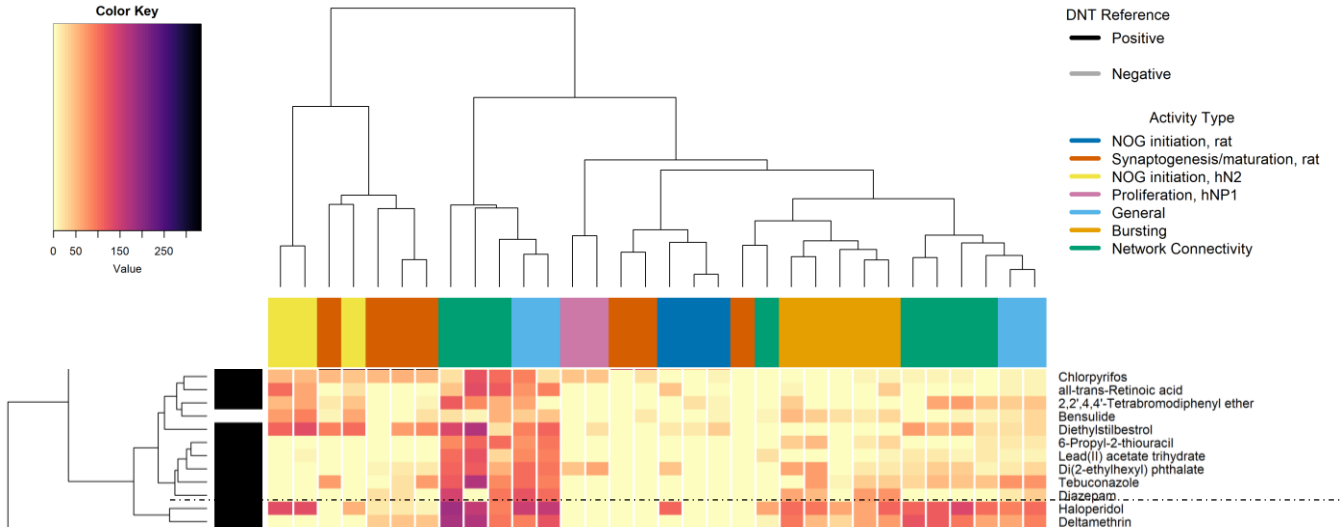


Cluster 2



High selectivity	Moderate/ Low selectivity
Network connectivity, general neuronal activity: -decreased network spike number -decreased mutual information	Proliferation
Bursting -decreased burst rate	Synaptogenesis/ neurite maturation
NOG (hN2)	NOG (rat cortical)

NOG: Neurite outgrowth

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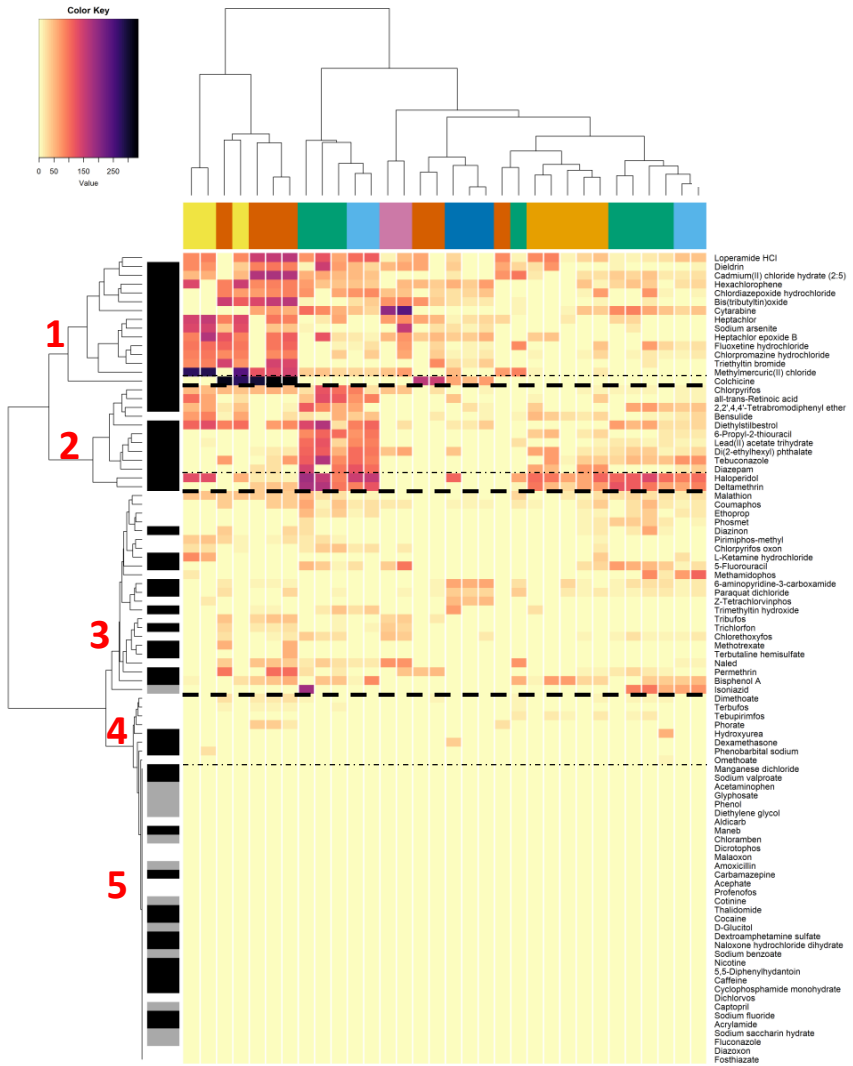

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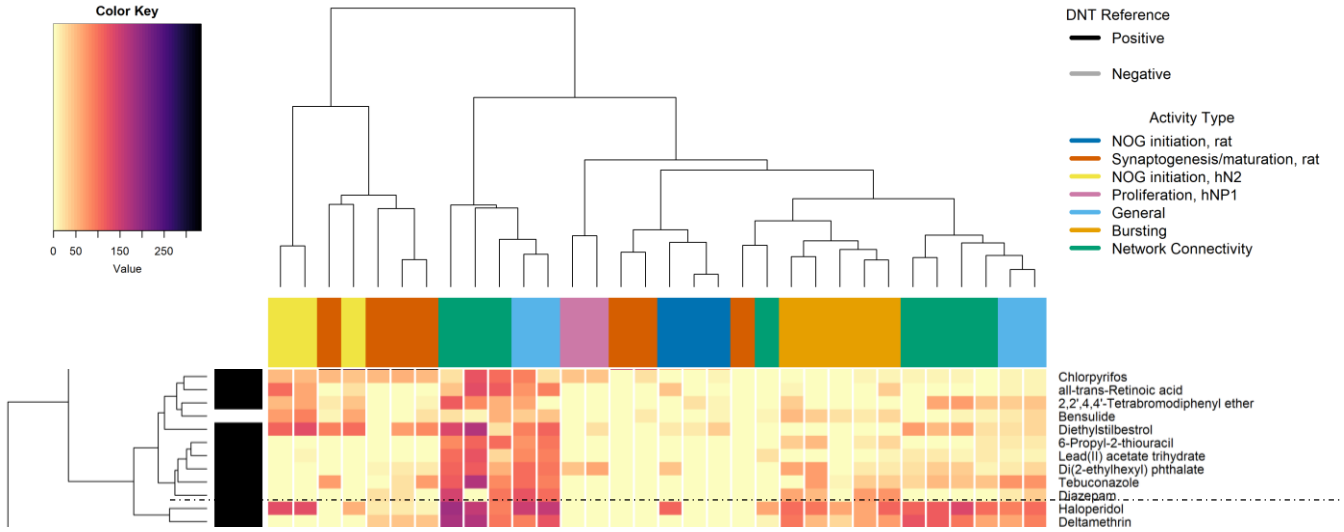


High selectivity	Moderate/ Low selectivity
Network connectivity, general neuronal activity	Proliferation
Bursting	Synaptogenesis/ neurite maturation
NOG (hN2)	NOG (rat cortical)

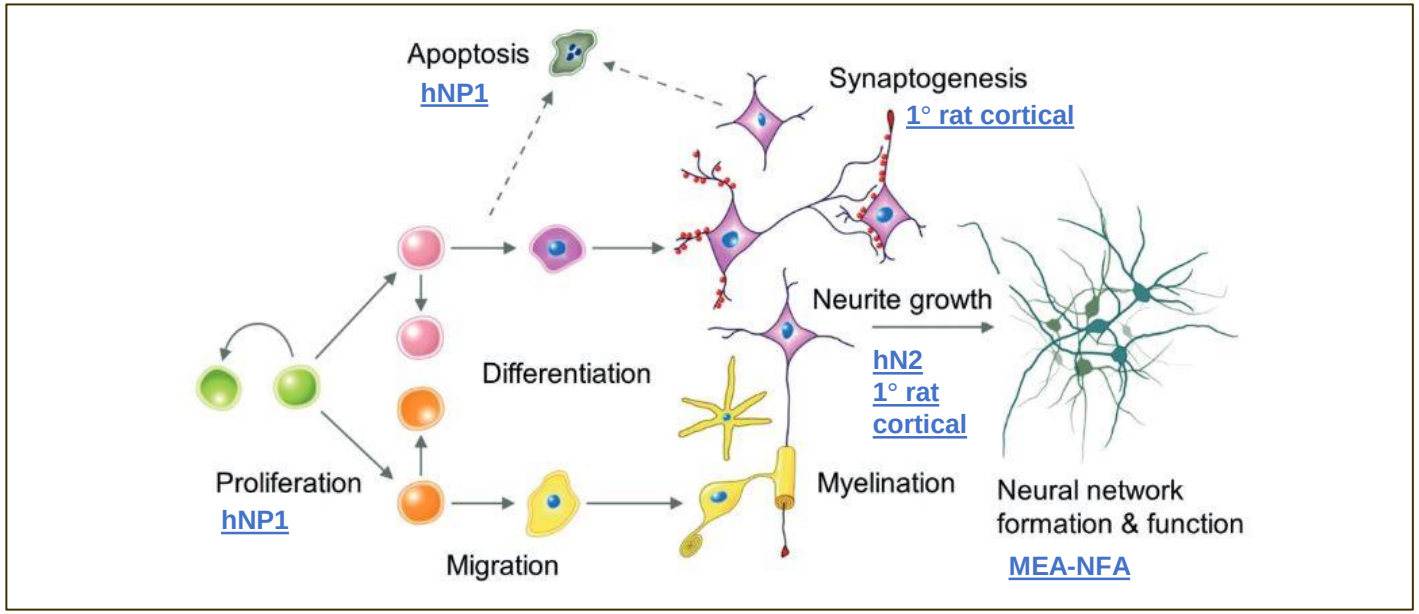
Haloperidol: antipsychotic, dopamine D_2 receptor antagonist

Deltamethrin: pyrethroid insecticide, voltage-gated sodium channels modulators

NOG: Neurite outgrowth

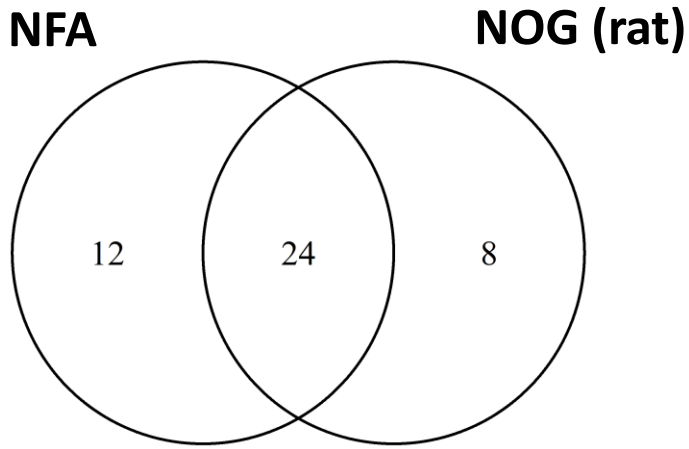
Trends in perturbations of key cellular processes

Do disruptions in early-stage processes (neurite maturation/synaptogenesis) correspond with disruptions in later-stage processes (network formation)?



Early-stage Developement → **Late-stage development**

Venn Diagram



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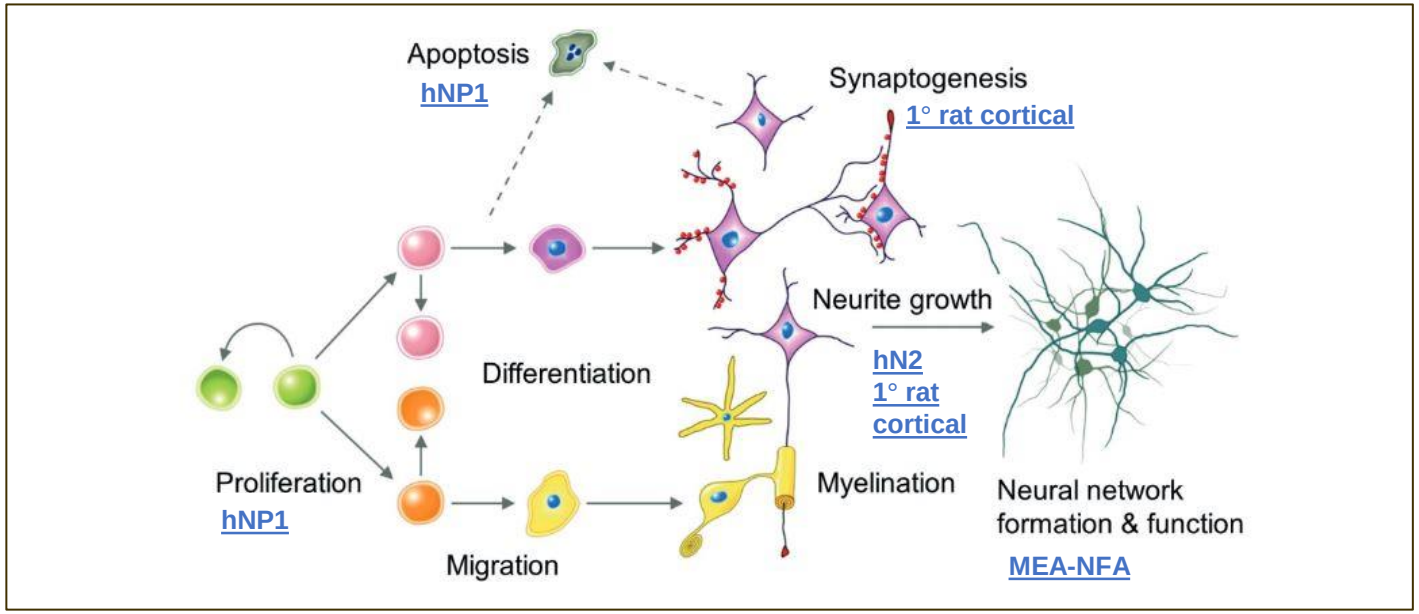

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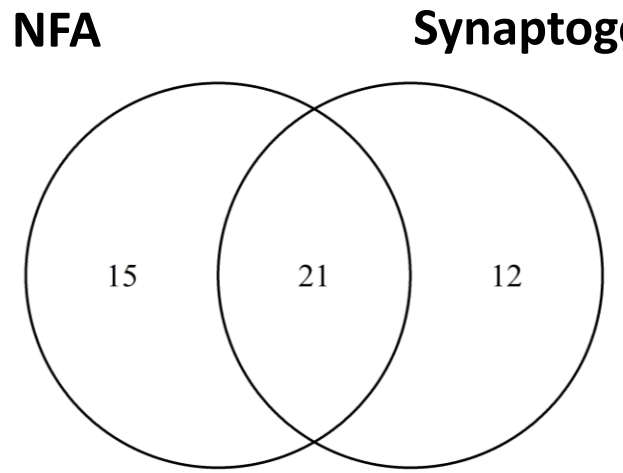

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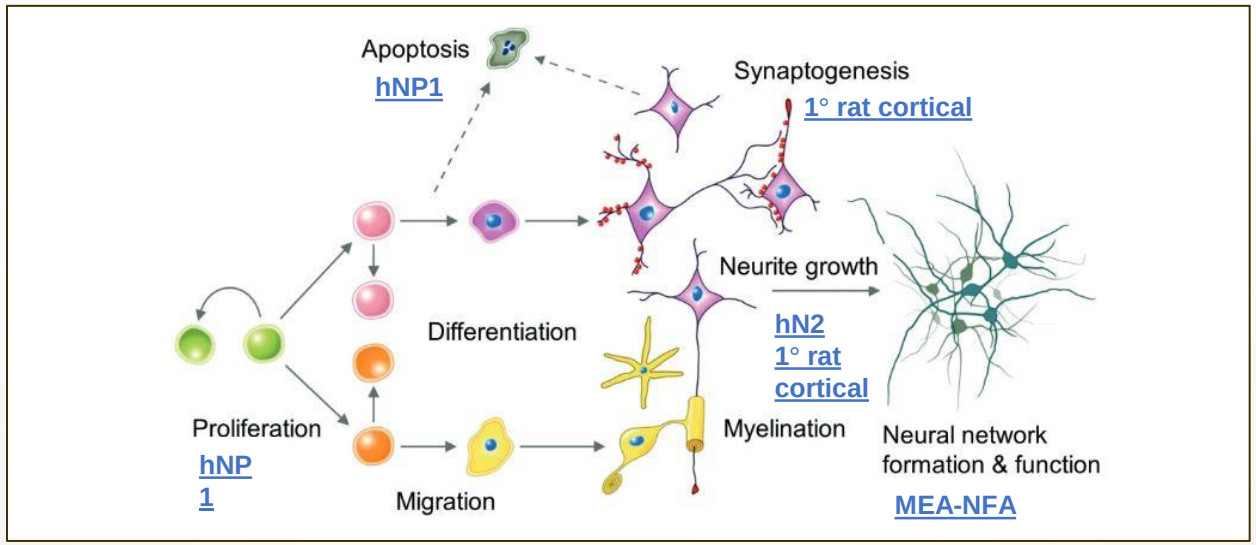
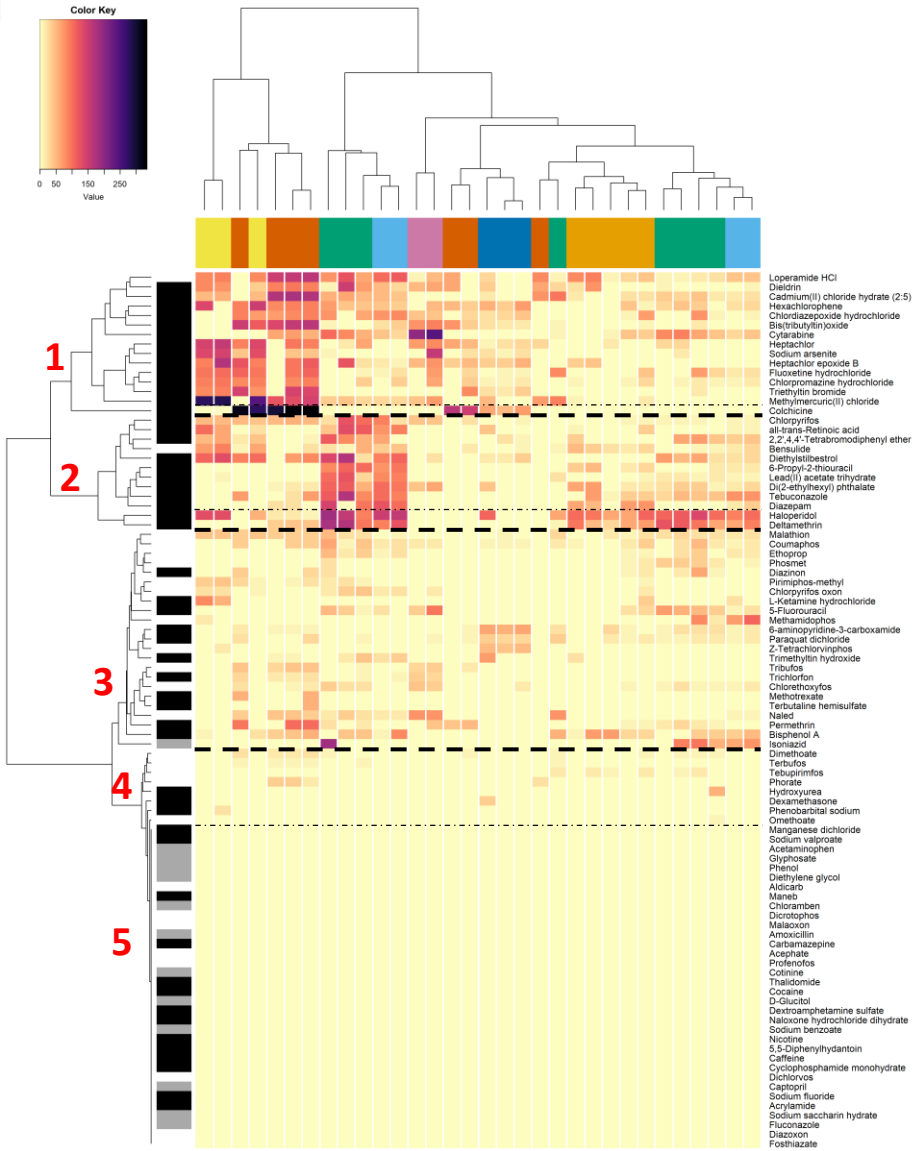


Can we use the DNT-NAM battery to classify DNT reference chemicals?



DNT reference

- Positive
- Negative

Activity Type

- NOG initiation, rat
- Synaptogenesis/maturation, rat
- NOG initiation, hN2
- Proliferation, hNP1
- General
- Bursting
- Network Connectivity

NOG: Neurite outgrowth

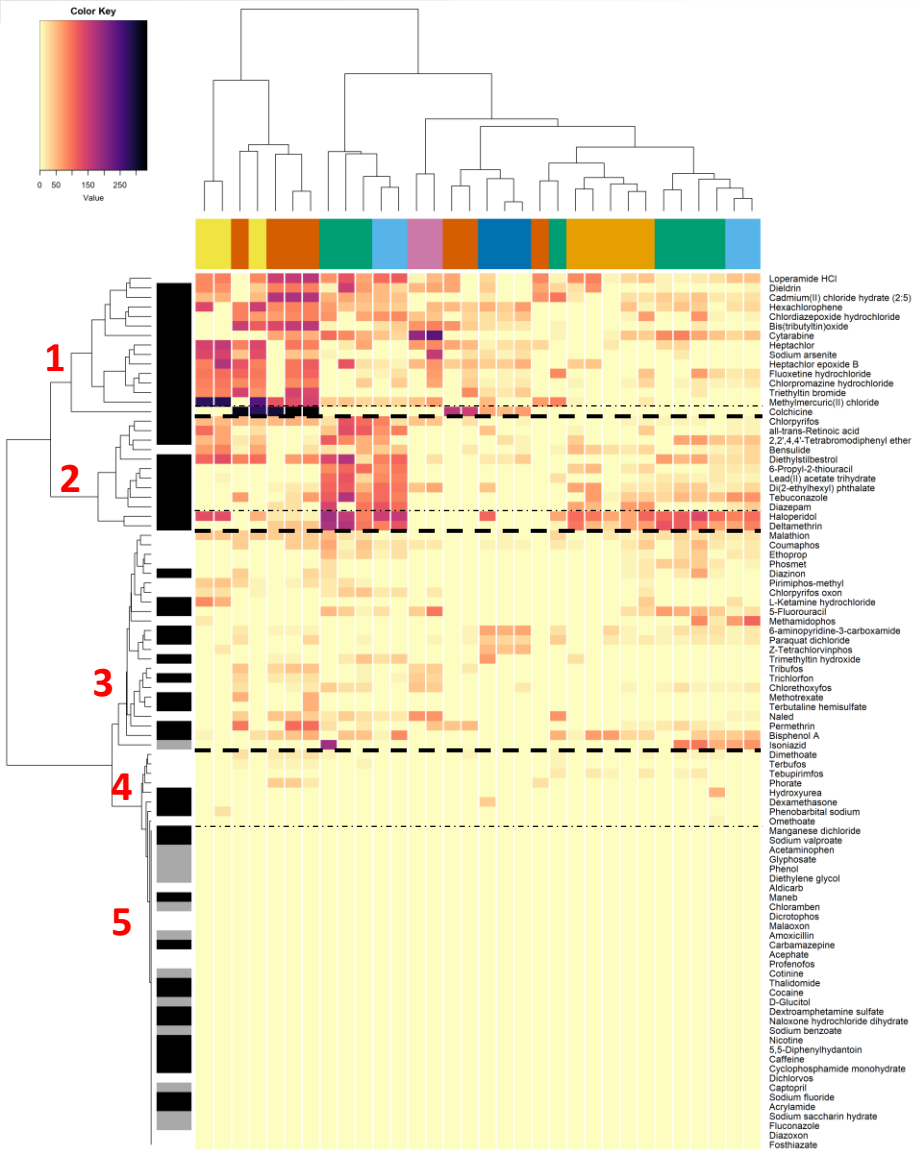
Reference DNT positives and negatives

Positives (53 matches)	
2,2',4,4'-Tetrabromodiphenyl ether	Fluoxetine hydrochloride
5,5-Diphenylhydantoin	Haloperidol
5-Fluorouracil	Heptachlor
6-aminopyridine-3-carboxamide	Heptachlor epoxide B
6-Propyl-2-thiouracil	Hexachlorophene
Acrylamide	Hydroxyurea
all-trans-Retinoic acid	Lead(II) acetate trihydrate
Bis(tributyltin)oxide	L-Ketamine hydrochloride
Bisphenol A	Maneb
Cadmium(II) chloride hydrate (2:5)	Manganese dichloride
Caffeine	Methotrexate
Carbamazepine	Methylmercuric(II) chloride
Chlordiazepoxide hydrochloride	Naloxone hydrochloride dihydrate
Chlorpromazine hydrochloride	Nicotine
Chlorpyrifos	Paraquat dichloride
Cocaine	Permethrin
Colchicine	Phenobarbital sodium
Cyclophosphamide monohydrate	Sodium arsenite
Cytarabine	Sodium fluoride
Deltamethrin	Sodium valproate
Dexamethasone	Tebuconazole
Dextroamphetamine sulfate	Terbutaline hemisulfate
Di(2-ethylhexyl) phthalate	Thalidomide
Diazepam	Trichlorfon
Diazinon	Triethyltin bromide
Dieldrin	Trimethyltin hydroxide
Diethylstilbestrol	

Negatives (13 matches)
Phenol
Amoxicillin
D-Glucitol
Sodium saccharin hydrate
Acetaminophen
Glyphosate
Isoniazid
Captopril
Diethylene glycol
Sodium benzoate
Cotinine
Chloramben
Fluconazole

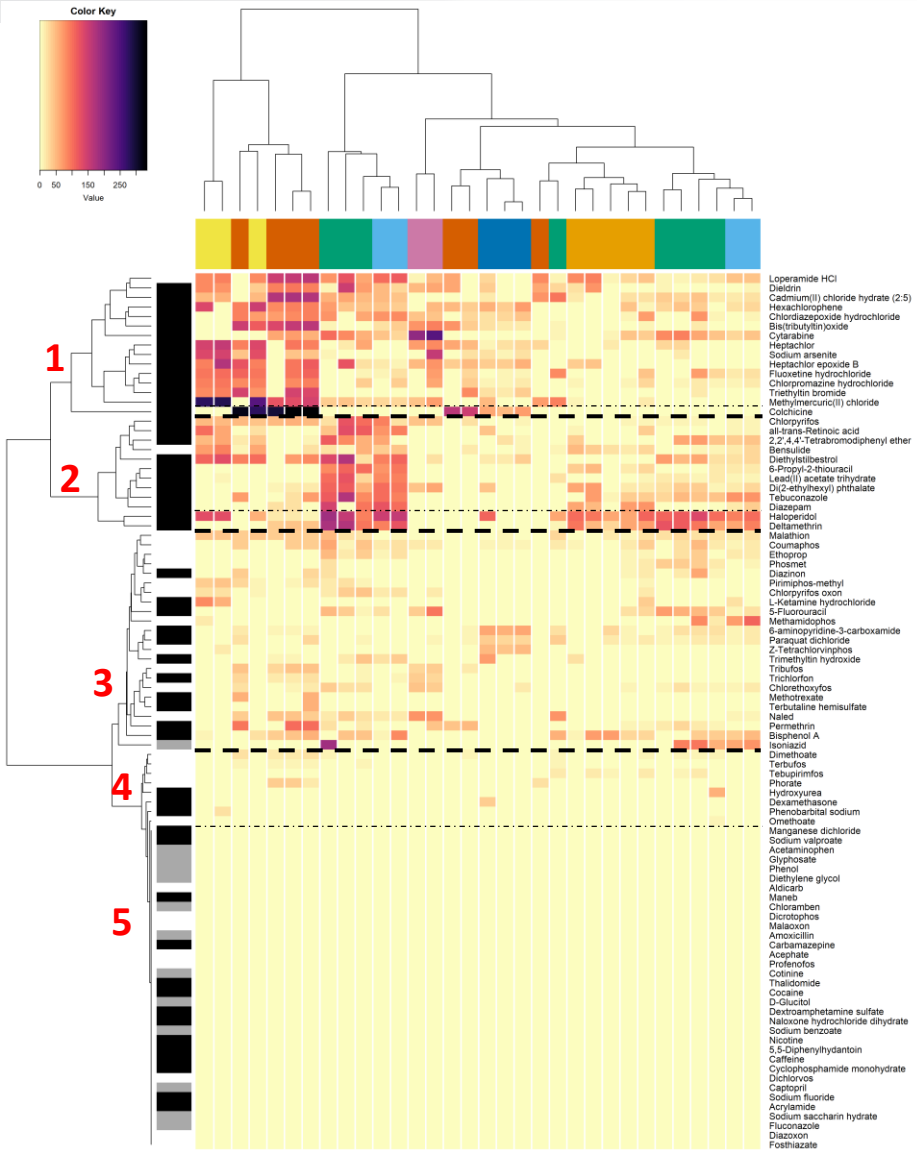
Mundy, et al. 2015. Neurotoxicology and Teratology
Harrill, et al., 2018. Toxicology and Applied Pharmacology

Can we use the DNT-NAM battery to classify DNT reference chemicals?



Classification		*In vivo* Reference chemicals	
		Negatives (13)	**Positives (53)**
	Cluster 1 **'High activity':** -Synaptogenesis/ proliferation/ NOG/ Neurite maturation	**0**	**14**
	Cluster 2 **'High activity'** -General/ network/ bursting activity/ synaptogenesis	**0**	**11**
	Cluster 3 **'Limited activity'** -General/ network activity/ bursting/ synaptogenesis/NOG	**1**	**11**
	Cluster 4 **'Highly selective'** -General/ network activity/ bursting/ synaptogenesis/NOG	**0**	**3**
	Cluster 5 **'Inactive/ equivocal'** -Increased network formation activity	**12**	**14**

Can we use the DNT-NAM battery to classify DNT reference chemicals?

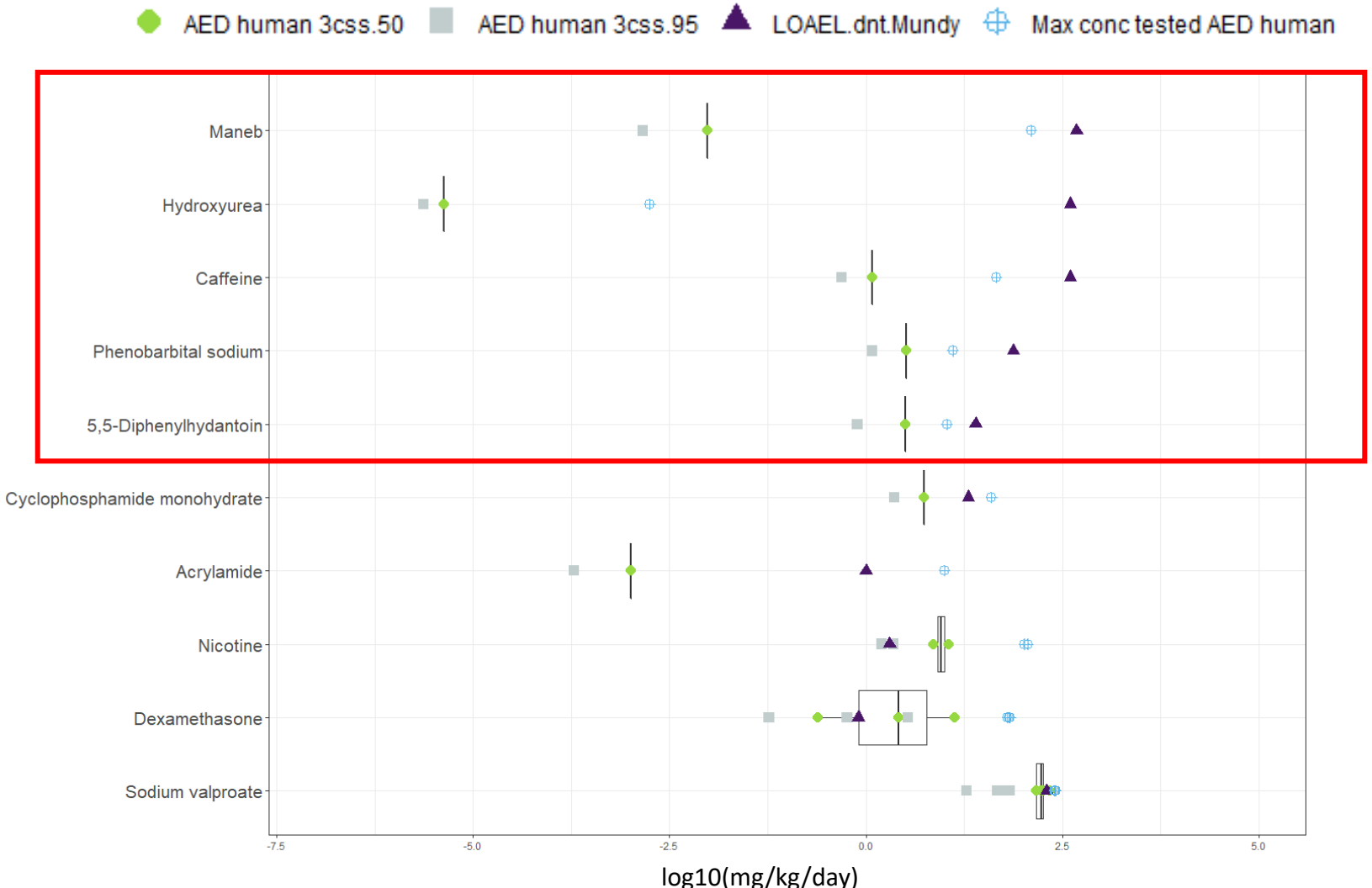


		Negatives	Positives
Results from DNT-NAM battery	**Selective activity** (Clusters 1,2,3,4)	False positive:1	True positive: 39
	Inactive/ equivocal (Cluster 5)	True Negative: 12	False negative: 14

Sensitivity= 74%, Specificity= 92%, Accuracy= 77%

False negatives: identifying gaps in the DNT-NAM battery

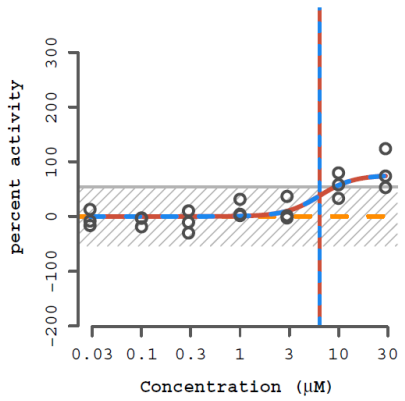
High-throughput toxicokinetic (**HTTK**) modeling to determine *in vitro* administered equivalent dose.



AED: administered equivalent dose
LOAEL: lowest observed adverse effect level

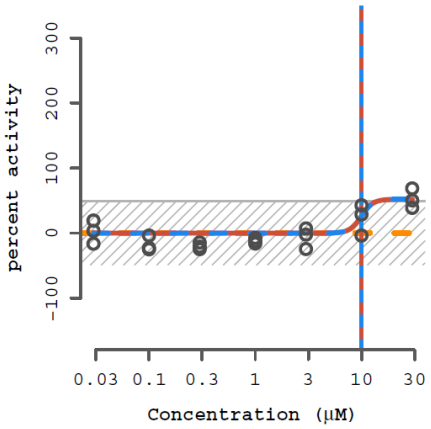
False negatives: identifying gaps in the DNT-NAM battery

Nicotine (1/2 hit)
Network_spike_spike_mean_up




```
ASSAY:    AEID2521 (CCTE_Shafer_MEA_dev_per_network_spik

NAME:     Nicotine
CHID:     20930    CASRN: 54-11-5
SPID(S): EX000416
M4ID:     41363188

HILL MODEL (in red):
       tp        ga        gw
val:   75.6      0.807     2.5
sd:    16.7      0.166     1.82

GAIN-LOSS MODEL (in blue):
       tp        ga        gw        la        lw
val:   75.6      0.807     2.5       2.97      10.9
sd:    NA        NA        NA        NA        NA

       CNST      HILL      GNLS
AIC:   216.97    192.92    196.92
PROB:  0         0.88      0.12
RMSE:  42.95     19.43     19.43

MAX_MEAN: 83.5      MAX_MED: 73.8      BMAD: 18

COFF: 54.1    HIT-CALL: 1    FITC: 41    ACTP: 1

FLAGS:
```

Nicotine (1/2 hit)
Correlation_coefficient_mean_up



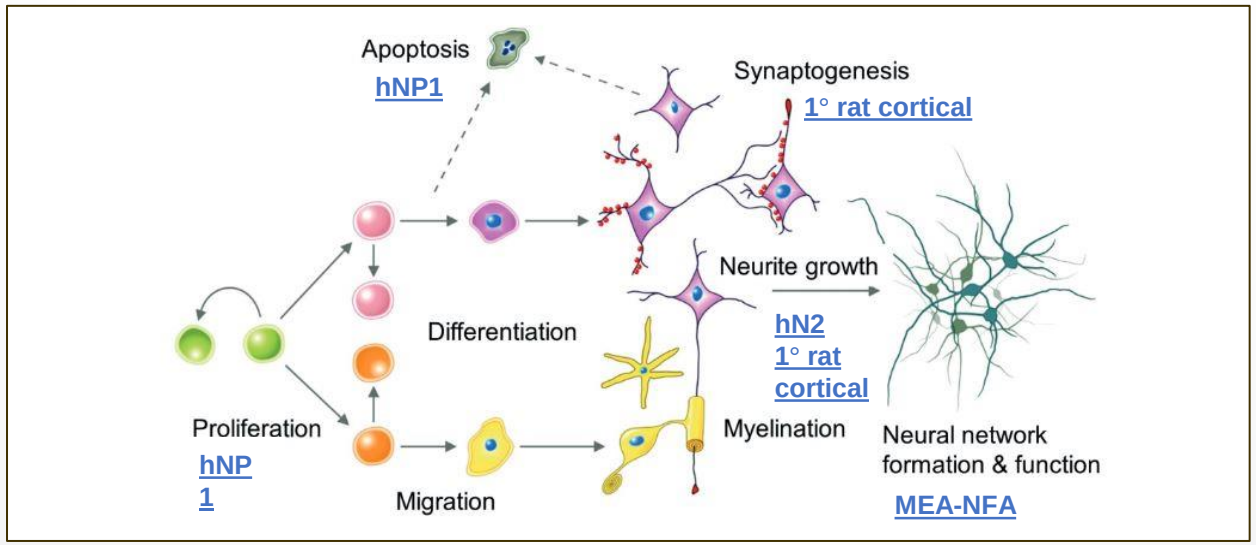

```
ASSAY:    AEID2525 (CCTE_Shafer_MEA_dev_correlation_coef

NAME:     Nicotine
CHID:     20930    CASRN: 54-11-5
SPID(S): EX000416
M4ID:     41364938

HILL MODEL (in red):
       tp        ga        gw
val:   52.1      0.999     8
sd:    9.68      0.0523    59.5

GAIN-LOSS MODEL (in blue):
       tp        ga        gw        la        lw
val:   52.7      1         8         2.13      2.9
sd:    NaN       NaN       NaN       NaN       NaN

       CNST      HILL      GNLS
AIC:   201.08    188.87    192.87
PROB:  0         0.88      0.12
RMSE:  27.26     16.64     16.64

MAX_MEAN: 52.6      MAX_MED: 50.4      BMAD: 16.3

COFF: 49      HIT-CALL: 1    FITC: 37    ACTP: 1

FLAGS: 11; 6
```

Aim:

Develop a battery of DNT-NAMs for fit-for-purpose evaluation of DNT.

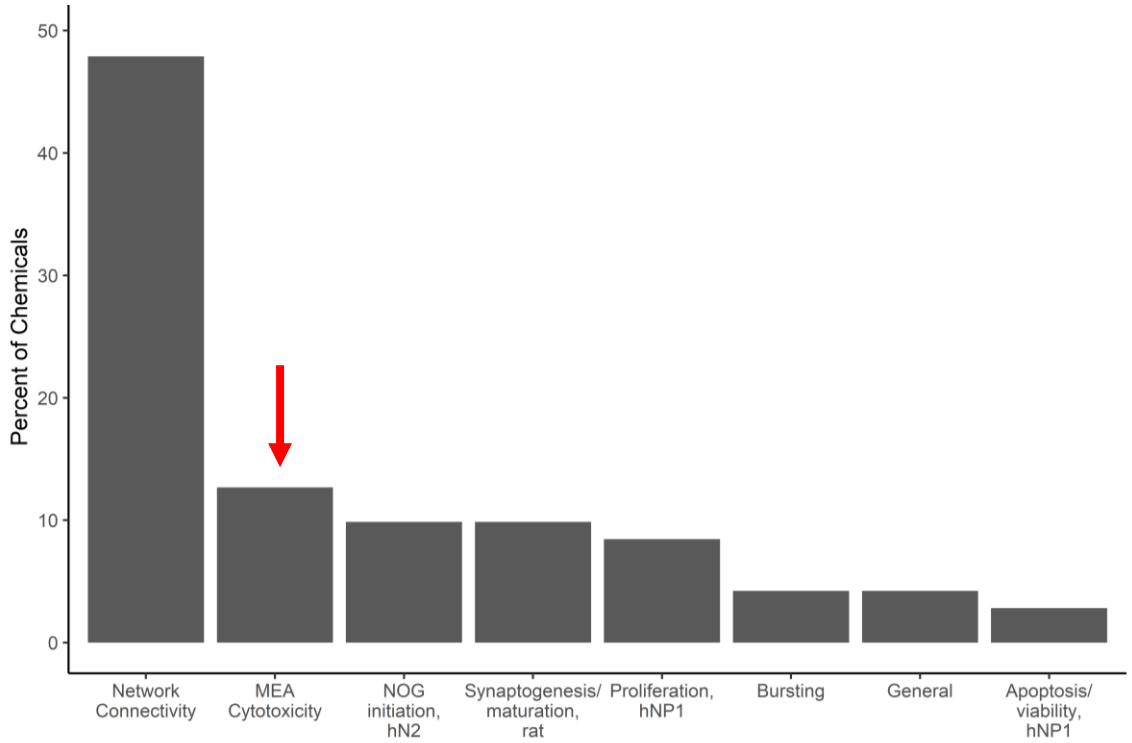
Questions:

1) How does the DNT-NAM battery collectively inform DNT-relevant bioactivity?
2) Does the DNT-NAM battery classify *in vivo* DNT reference chemicals?
3) Can we use the DNT-NAM battery to identify the most sensitive endpoints?

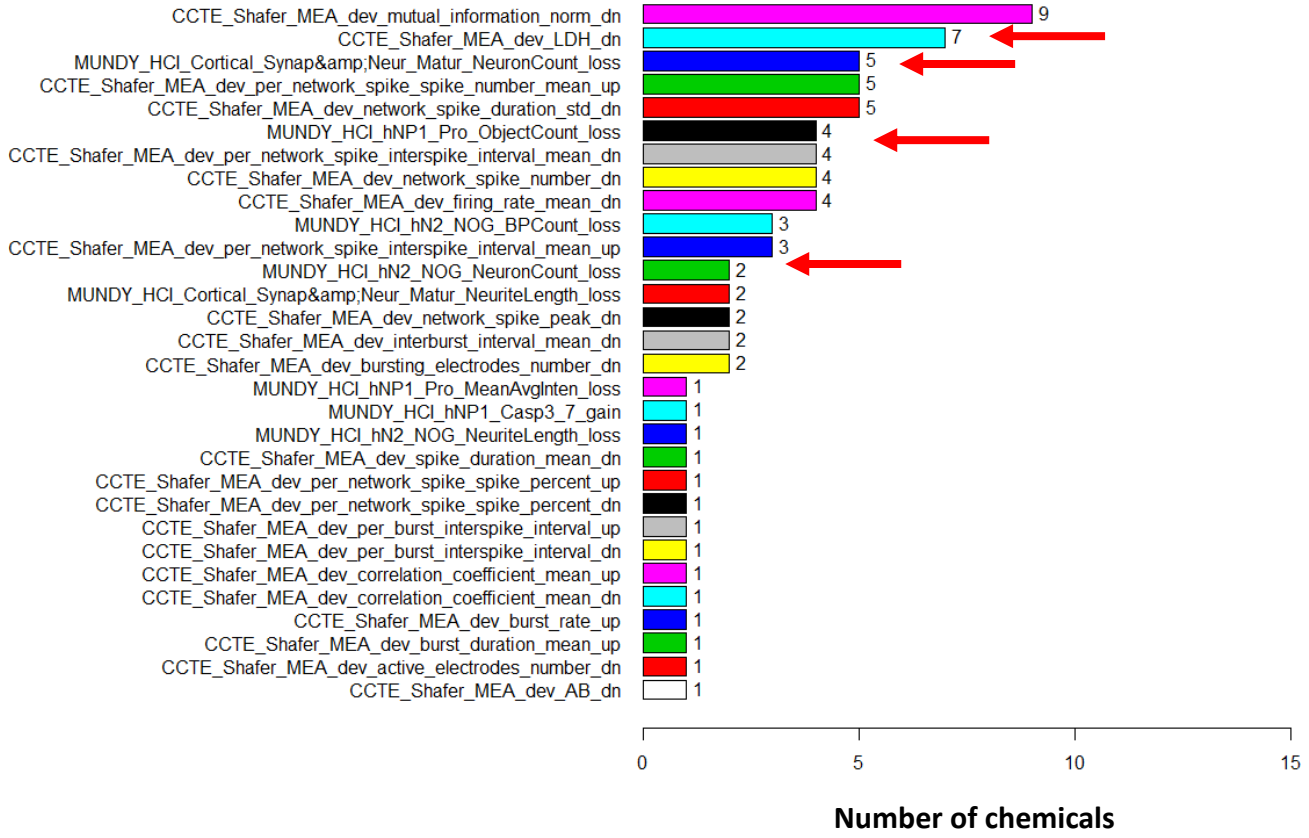


Network connectivity is the most sensitive ‘activity type’ in the DNT-NAM battery.

Minimum potency by activity type

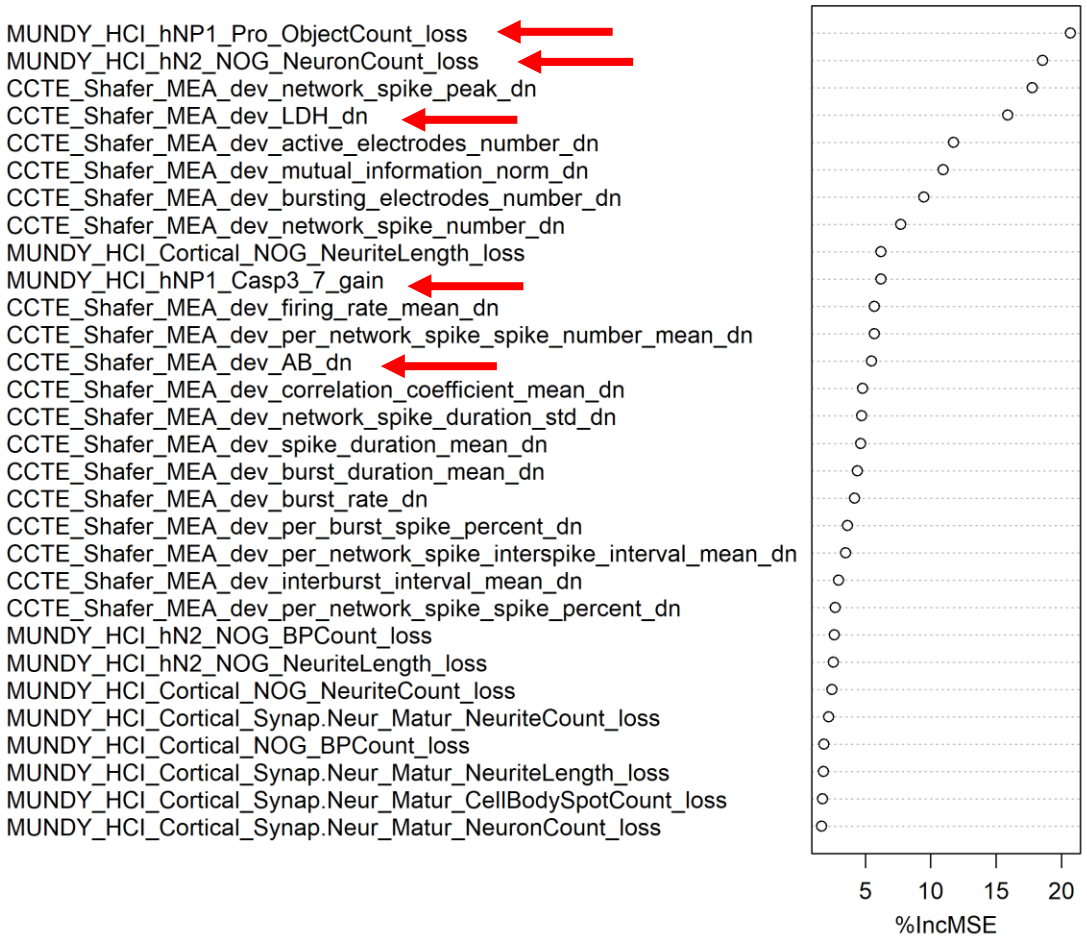


Minimum potency by endpoint

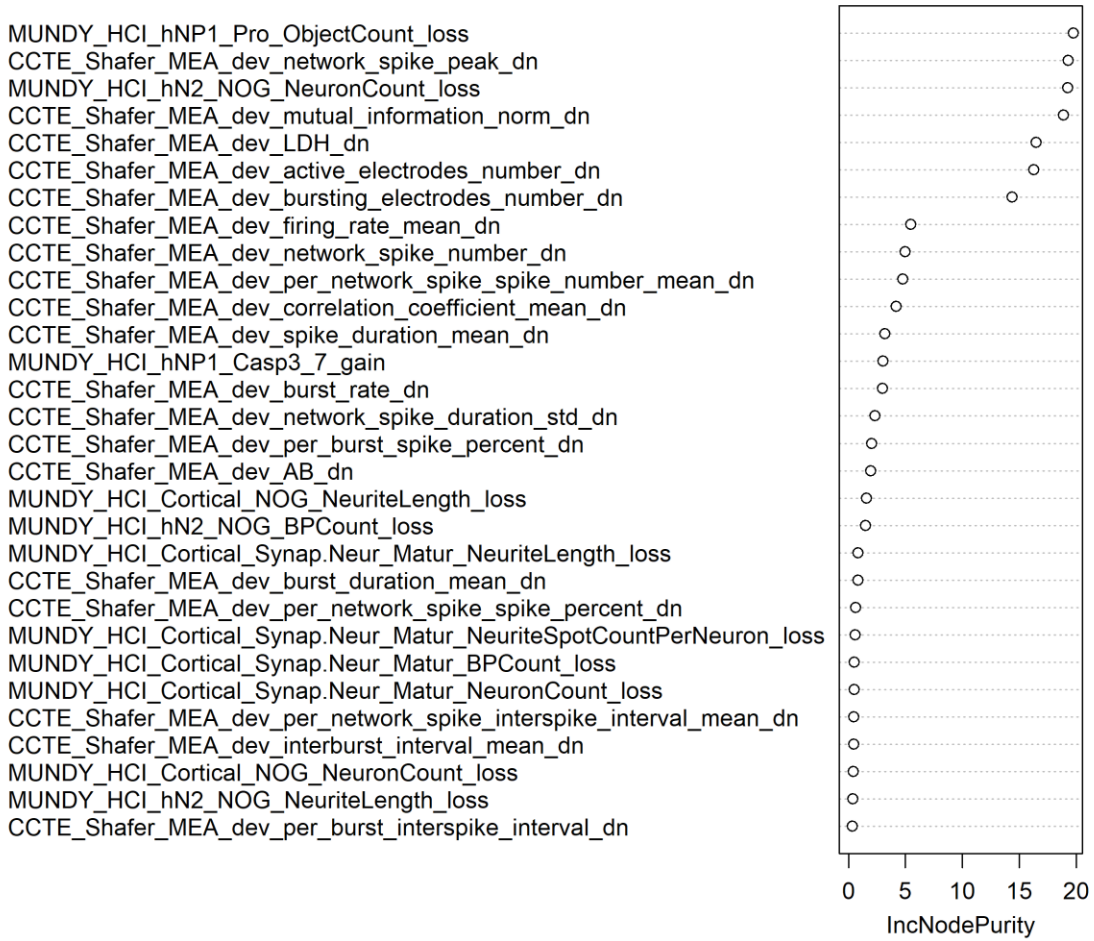


Random forest regression approach:
The 'most informative' endpoints in predicting minimum potency is cytotoxicity (hNP1).

Most important endpoint (mean square error)

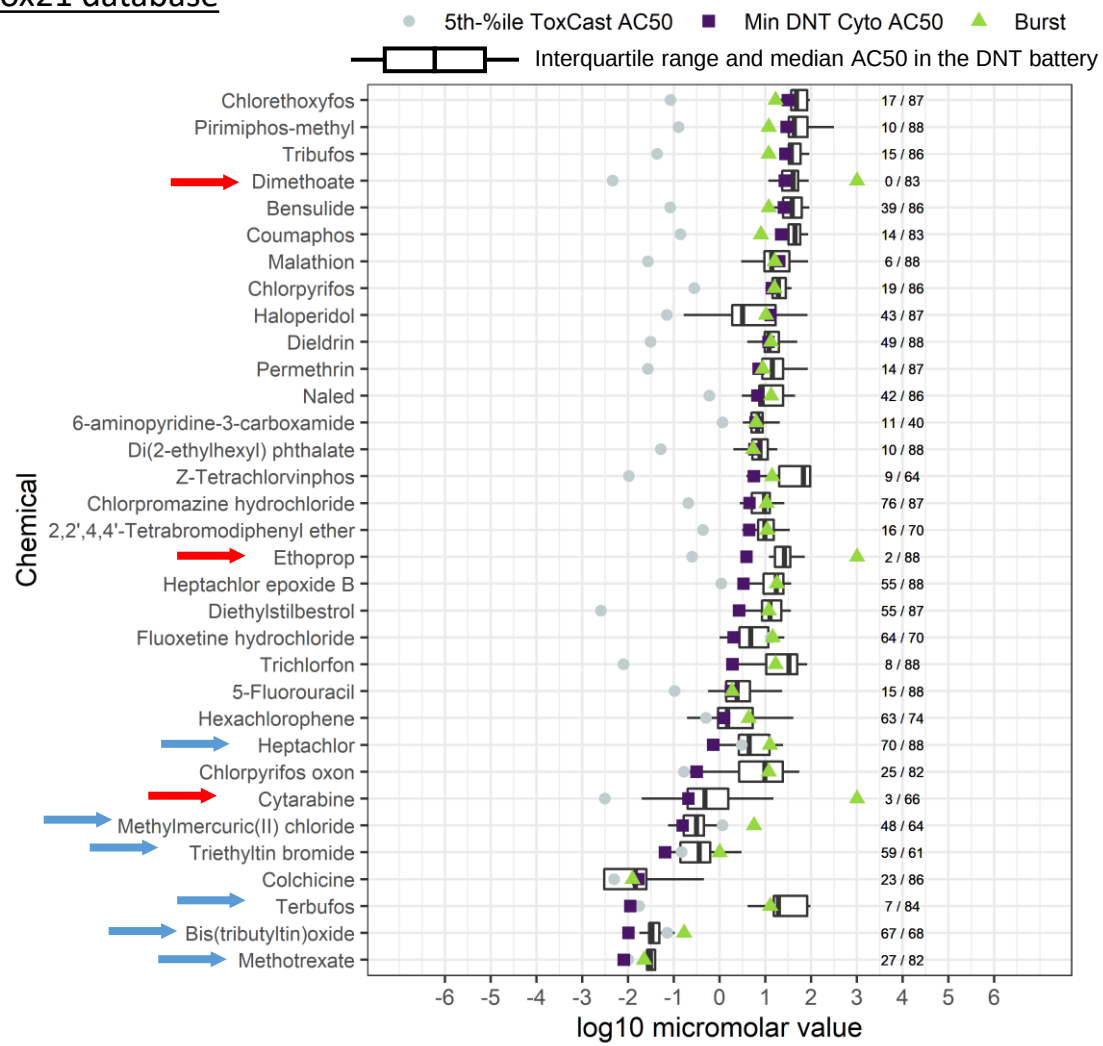


Most important endpoint (Node purity)



How does the minimum cytotoxicity in the DNT NAM battery compare to non-neuronal cell types (ToxCast/ Tox21 database)?

A comparison to ToxCast/ Tox21 database



Conclusions

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

- Log10-AC_{50} may not fully capture DNT-relevant bioactivity due to cytotoxicity effects.
- Chemical clusters are driven by **selective** activity in distinct cellular events and/or neuronal cell-types.
- Perturbations in upstream developmental cellular events (eg. synaptogenesis) does not predict perturbations in late-developmental cellular events (eg. network formation activity).

2) Does the DNT-NAM battery classify *in vivo* DNT reference chemicals?

- Using the selectivity metric, DNT reference chemicals are classified with high specificity and moderate sensitivity.
- False negatives provide insight into experimental and biological limitations.

3) Can we use the DNT-NAM battery to identify the most sensitive endpoints?

- Endpoints measuring decreased network connectivity in the MEA NFA and endpoints measuring cytotoxicity in multiple assays were identified as the most sensitive endpoints in predicting *in vitro* DNT.

Questions?

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