Linking DNT In Vitro Battery Endpoints to Adverse Outcome Pathways using Omics Approaches

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This work has been funded by the US. Environmental Protection Agency. I have no conflicts to declare.
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Alignment of DNT IVB assays with AOPs

Contents lists available at ScienceDirect

Neurotoxicology

journal homepage: www.elsevier.com/locate/neuro

Identification of neurotoxicology (NT)/developmental neurotoxicology (DNT) adverse outcome pathways and key event linkages with *in vitro* DNT screening assays

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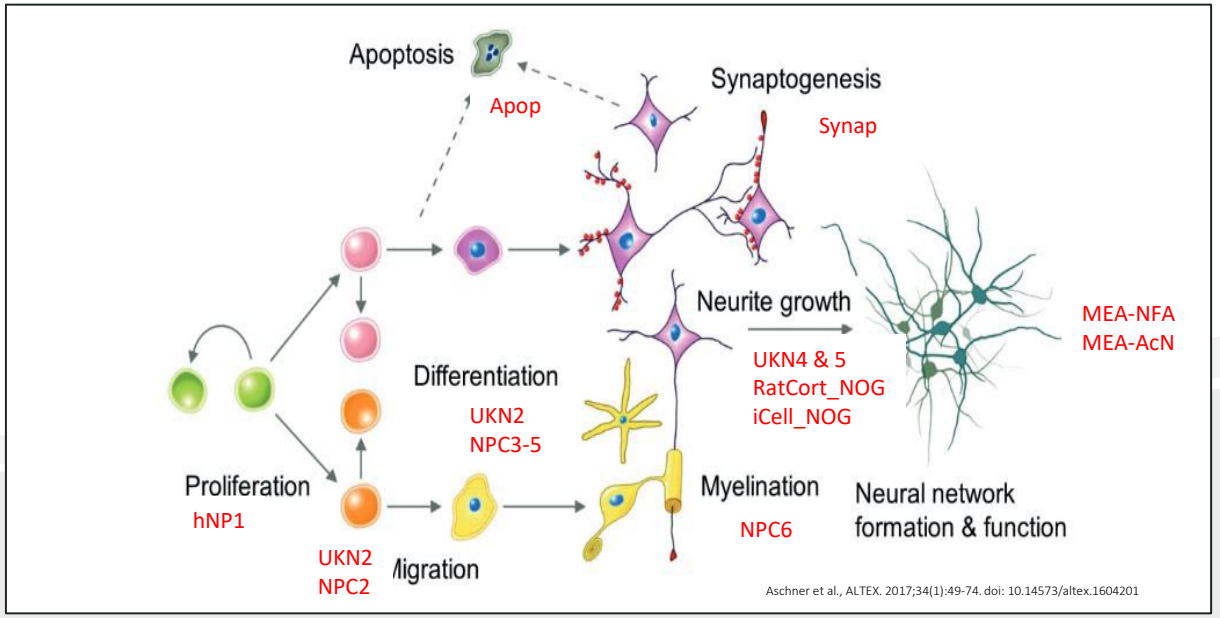
Archives of Toxicology (2019) 93:2759–2772
https://doi.org/10.1007/s00204-019-02551-1

REGULATORY TOXICOLOGY

Development and analysis of an adverse outcome pathway network for human neurotoxicity

Nicoleta Spinu[1] · Anna Bal-Price[2] · Mark T. D. Cronin[1] · Steven J. Enoch[1] · Judith C. Madden[1] · Andrew P. Worth[2]

Received: 15 June 2019 / Accepted: 14 August 2019 / Published online: 23 August 2019



Aligning different data streams to the AOP concept

Toxicant

Chemical Property Profile

Molecular Initiating Event

?

(many postulated, few confirmed)

Cellular Responses

Key Events

proliferation
differentiation
migration
neurite growth
synaptogenesis

Organ Responses

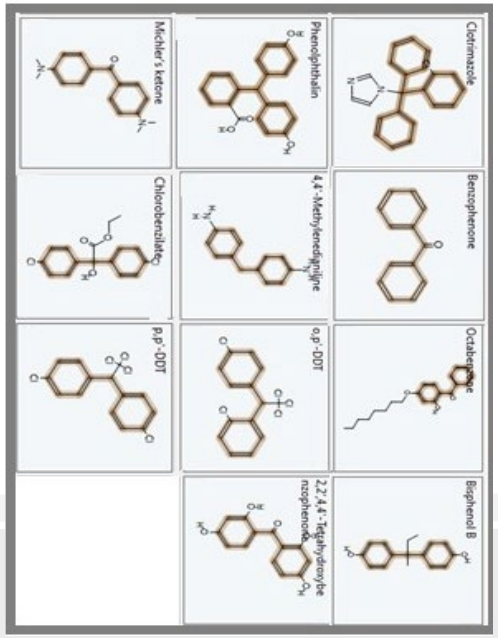
Nervous System

Δ connectivity (morphology, neurochemistry, physiology)

Individual Responses

behavior
cognition

"Chemotyping"



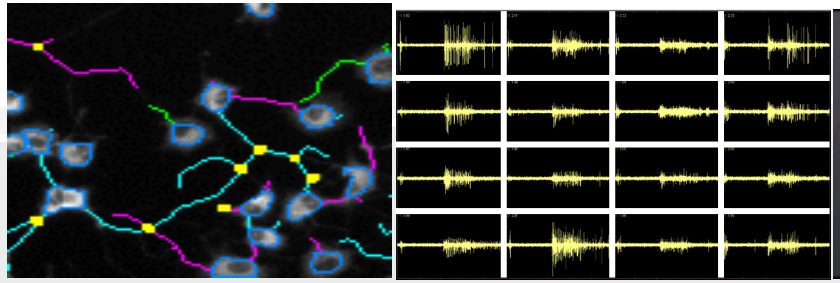
Target-based Screening (known MIEs)

HTP Molecular assays (ToxCast)

ion channels
AChE
neurotransmitter receptors
growth factor receptors
transcription factors
kinases
…

Phenotypic Screening in physiologically-relevant models (MIE not assumed)

***In Vitro* Approaches**



Behavior

Linking DNT-NAMs data to AOPs

- Few endorsed AOPs exist for DNT
  - Especially if thyroid related AOPs are excluded
  - These AOPs are not necessarily driven by data from the DNT NAMs

- Is there another approach that would use data to establish linkages between data from DNT NAMs and AOPs for neurodevelopment?
  - Could this also help to establish human relevance?

Could Omics data help link data from in vitro DNT assays to AOPs?

Toxicology and Applied Pharmacology 354 (2018) 81–93

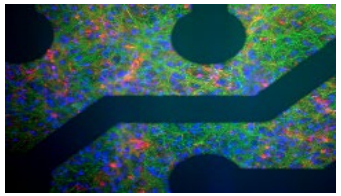
Contents lists available at ScienceDirect

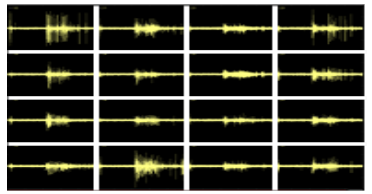
Toxicology and Applied Pharmacology

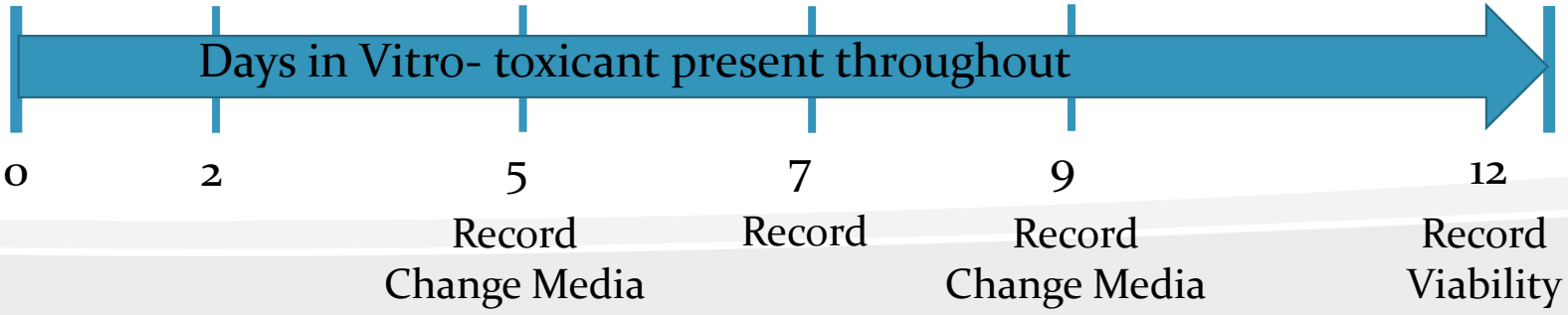
journal homepage: www.elsevier.com/locate/taap

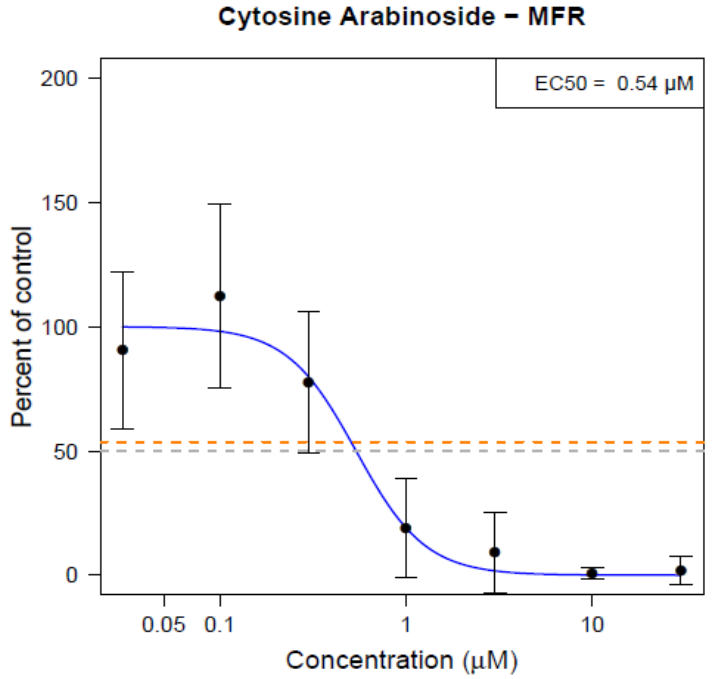
Defining toxicological tipping points in neuronal network development☆

Christopher L. Frank[a,1], Jasmine P. Brown[a,2], Kathleen Wallace[a], John F. Wambaugh[b], Imran Shah[b], Timothy J. Shafer[a,*]



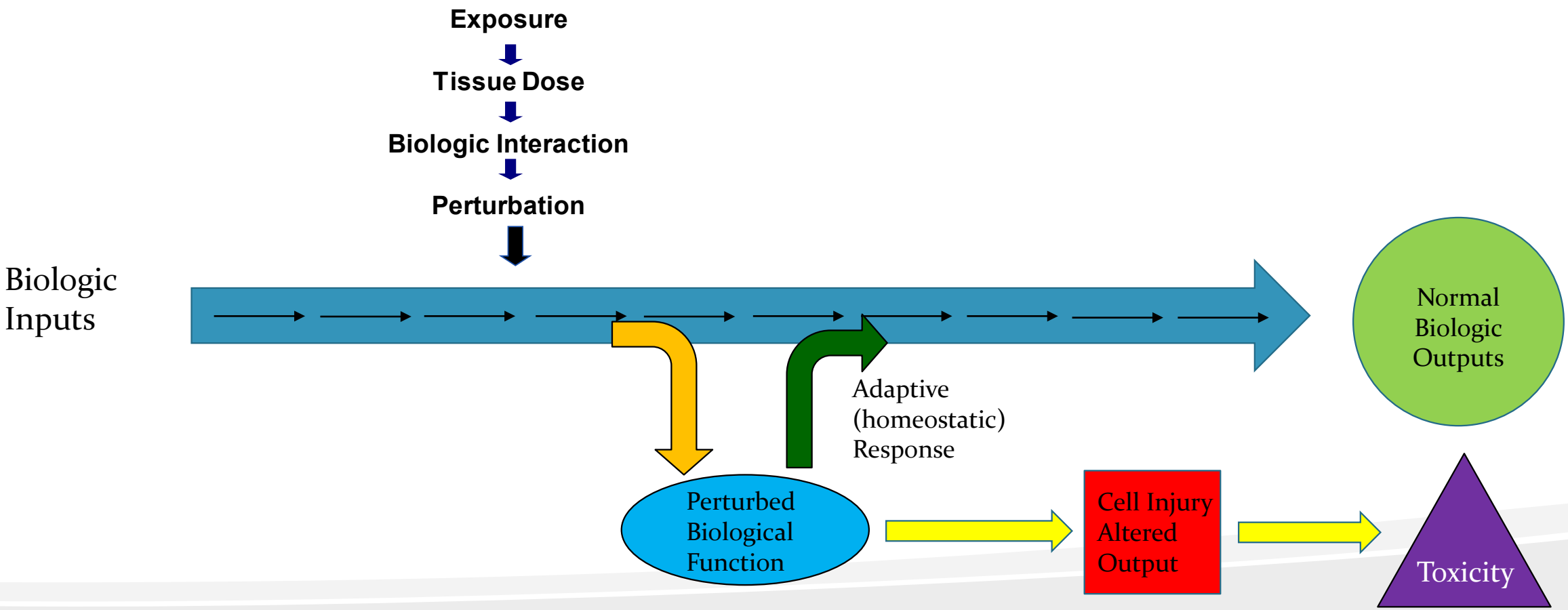






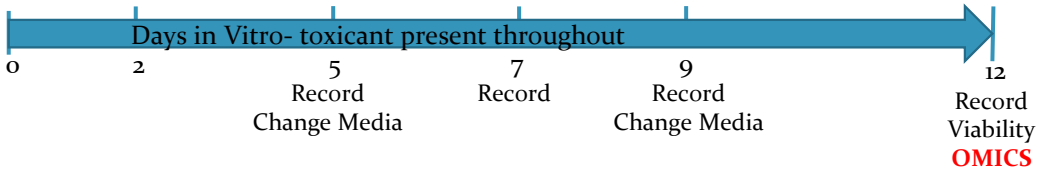
Critical concentration ("tipping point") determined =0.046 µM for Cytosine Arabinoside

The Concept of Toxicological "Tipping Points"

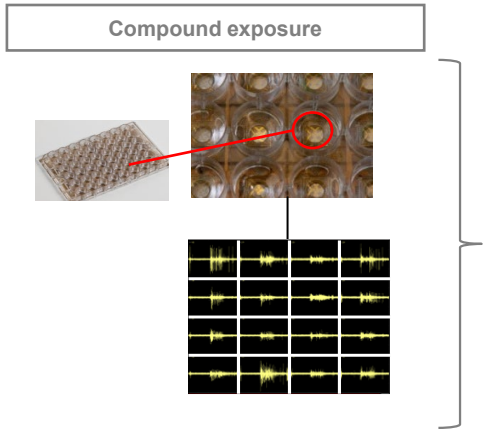


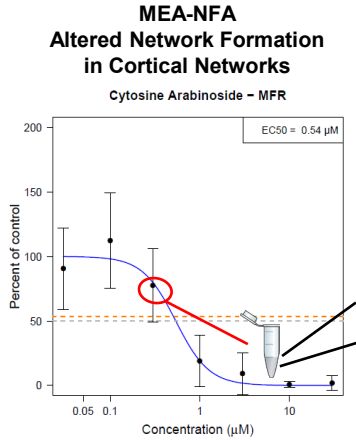
The "Tipping Point" is a mathematical determination of the point at which the system transitions from homeostasis to toxicity

Proof-of-Concept for collecting –omics information from the rat network formation assay



Step 1: Chemical Dose Identification with Functional Assay

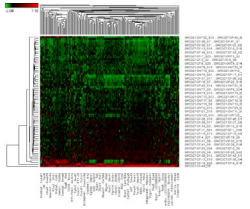




Selected Treatments

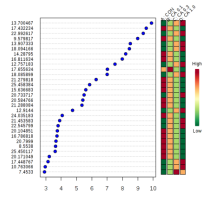
Step 2: Identifying Key Molecular Events Involved in Neurodevelopment

Cell Pellet
media



Transcriptomics

+



Metabolomics

3: Integrated Core Analysis

Transcriptomic level

Disease Analysis (NDD)
Molecular/Cellular
Function Categories

Metabolomic level

Disease Analysis (NDD)
Molecular/Cellular
Function Categories

Combined Analysis

Disease Analysis (NDD)
Molecular/Cellular
Function Categories

Marable et al., Toxicol Sci. 2022 Feb 28;186(1):118-133. doi: 10.1093/toxsci/kfab151

Proof-of-Concept for collecting –omics information from the rat network formation assay

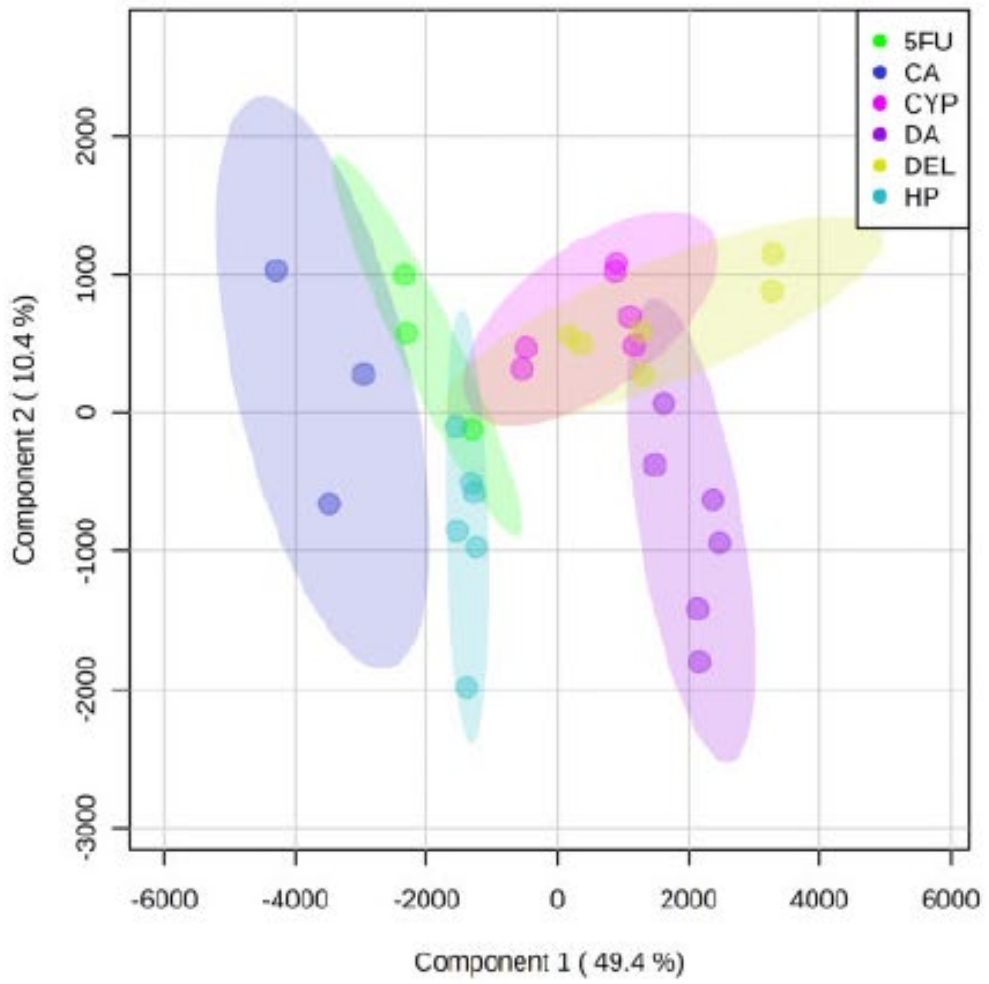
Table 1. Compounds Tested

Compound Name (Abbreviation)	Proposed Target	CAS No.	DTXSID	Tipping Point[a] (μM [±95% CI])	Concentrations Tested (μM)	Min Viability[b] EC_{50} (μM)	Solvent Used	Source
Cytosine arabinoside (CA)	DNA synthesis inhibitor	147-94-4	DTXSID3022877	0.046 [0.04–0.06]	1	0.84	DMSO	Sigma-Aldrich
5-Fluorouracil (5FU)	DNA synthesis inhibitor	51-21-8	DTXSID2020634	0.14 [0.10–0.17]	1	2.28	DMSO	Sigma-Aldrich
Domoic acid (DA)	Glutamate Receptor Agonist	14277-97-5	DTXSID20274180	0.21 [0.9–0.25]	0.3	NA	Water	Sigma-Aldrich
Cypermethrin (CM)	Voltage-gated Sodium Channel Modulator	52315-07-8	DTXSID1023998	0.28 [0.19–0.38]	3.10	NA	DMSO	Chem Service, Inc.
Deltamethrin (DM)	Voltage-gated Sodium Channel Modulator	52918-63-5	DTXSID8020381	0.05 [0.04–0.15]	3.10	NA	DMSO	Chem Service, Inc.
Haloperidol (HP)	Dopamine Receptor Agonist	52-86-8	DTXSID4034150	0.31 [0.15–0.49]	3	13.9	DMSO	Sigma-Aldrich

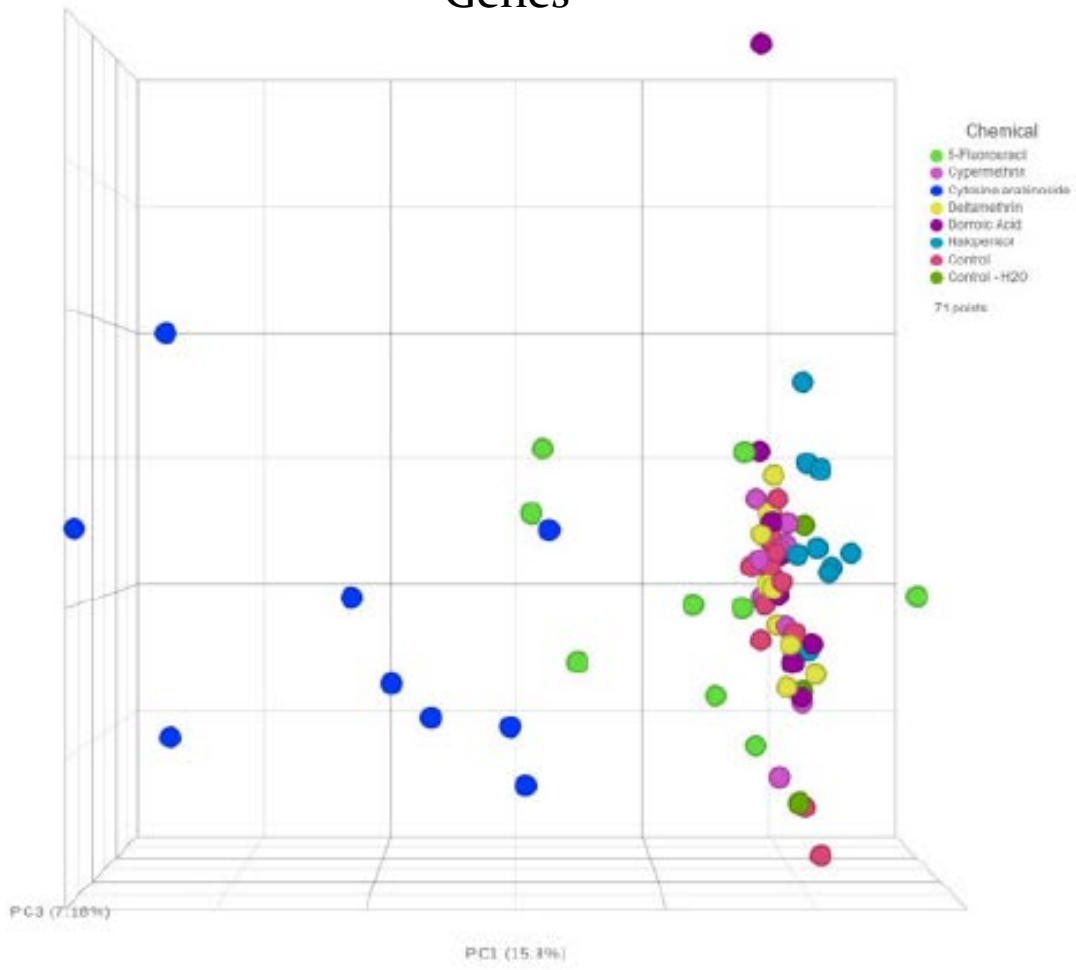
Marable et al., Toxicol Sci. 2022 Feb 28;186(1):118-133. doi: 10.1093/toxsci/kfab151

Principal Components Analysis for Differentially Expressed Genes and Metabolites indicate treatment effects

Metabolites



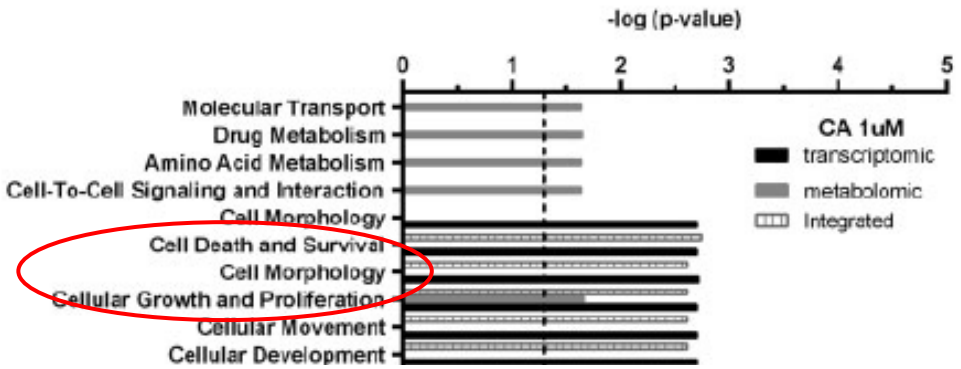
Genes

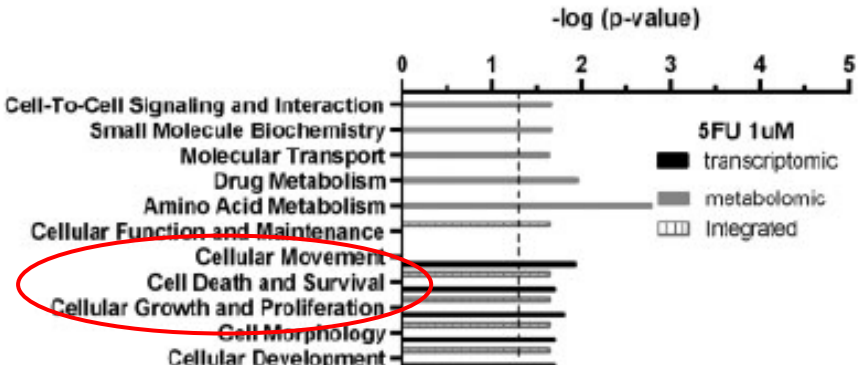


Marable et al., Toxicol Sci. 2022 Feb 28;186(1):118-133. doi: 10.1093/toxsci/kfab151

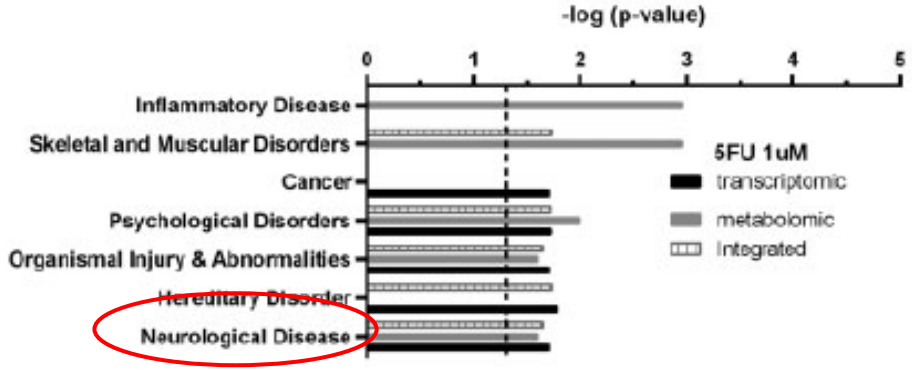
Categories Associated With Functional Alterations in Network Development

Cellular and Molecular Function





Disease and Disorders



Marable et al., Toxicol Sci. 2022 Feb 28;186(1):118-133. doi: 10.1093/toxsci/kfab151

Canonical Pathways Associated With Functional Alterations in Network Development

Table 3. Canonical Pathways Associated With Altered Network Formation Using Integrated Pathway Analysis

Cytosine Arabinoside (CA)	5-Fluorouracil (5FU)	Domoic Acid (DA)	Cypermethrin (CM)	Deltamethrin (DM)	Haloperidol (HP)
• CREB signaling in neurons	• WNT/β-catenin signaling	• tRNA charging	• Alanine biosynthesis III	• (S)-reticuline biosynthesis II	• Phenylalanine degradation I
• Axonal guidance signaling	• Axonal guidance signaling	• Phenylalanine degradation IV (mammalian, via side chain)	• Glycine biosynthesis III	• Tyrosine degradation I	• Threonine degradation II
• Osteoarthritis pathway	• tRNA charging	• Glycine biosynthesis III	• Catecholamine biosynthesis	• 4-hydroxybenzoate biosynthesis	• Tyrosine biosynthesis IV
• Human embryonic stem cell pluripotency	• Hepatocyte Growth Factor (HGF) signaling	• Purine nucleotides de novo biosynthesis II	• tRNA charging	• 4-hydroxyphenylpyruvate biosynthesis	• Glycine biosynthesis III
• Hepatic fibrosis/ hepatic stellate cell activation	• Nicotinamide adenine dinucleotide (NAD) signaling pathway	• Tyrosine biosynthesis IV	• Glycine Biosynthesis III	• tRNA charging	• tRNA charging
	• Hepatic fibrosis/ hepatic stellate cell activation		• Thio-molybdenum cofactor biosynthesis		

Marable et al., Toxicol Sci. 2022 Feb 28;186(1):118-133. doi: 10.1093/toxsci/kfab151

Upstream Regulators Associated with Functional Alterations in Network Development

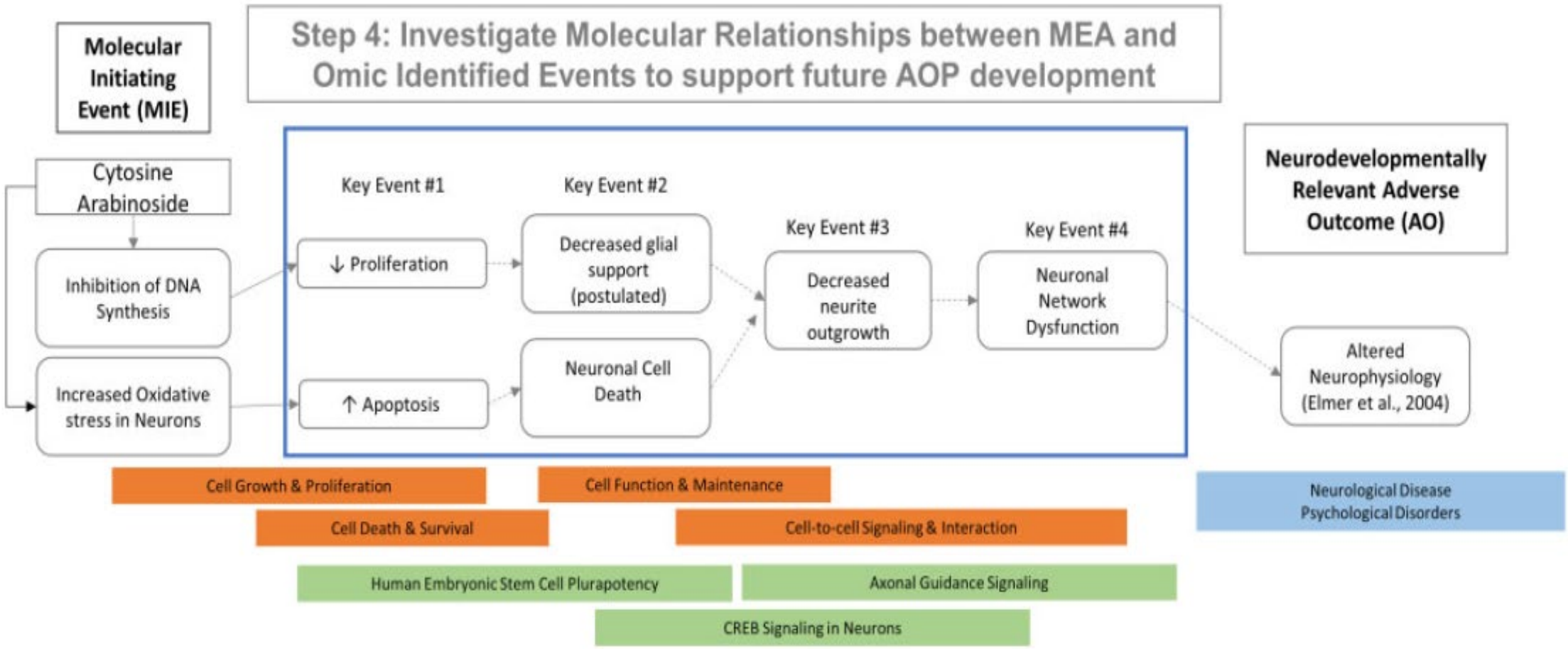
Table 4. Top Upstream Regulators Associated with Chemical Exposures That Alter Network Formation

	Cytosine Arabinoside (CA)	5-Fluorouracil (5FU)	Domoic Acid (DA)	Cypermethrin (CM)	Deltamethrin (DM)	Haloperidol (HP)
Transcriptomic regulators	CREB1↑	SOX2↓	LOC102724788/ PRODH	ARX	ATXN1	CREB1
	SOX2↓	TCF7L2↓	SLC1A2	LOC102724788/ PRODH	GLS	CA9
	ZBTB17	PRMT1↓	ELAVL4	SLC1A2	HPRT1	CPT1B
	S100A8↑	PPP3CA	GLS	KLF9	ELAVL4	
		NOTCH1		SLC23A2		
Metabolomic regulators	β-estradiol		Kynurenic acid		K+	Afatinb
						GSKJ4

Predicted activation (↑) and inhibition (↓). Lack of a directional arrow indicates that activation or inhibition could not be predicted.

Marable et al., Toxicol Sci. 2022 Feb 28;186(1):118-133. doi: 10.1093/toxsci/kfab151

A Putative AOP based on NAMs and Omics Data



Marable et al., Toxicol Sci. 2022 Feb 28;186(1):118-133. doi: 10.1093/toxsci/kfab151

Next Step: Confirm with additional data

Chemical	Functional Category	CAS No.	DTXSID	Tipping Point⁺ (µM [±95% CI])	Concentrations Tested (µM) LOW/MID/HIGH	Min AC_{50} Endpoint*	Min AC_{50} Viability (µM)*
Tebuconazole	Fungicides	107534-96-3	DTXSID9032113	1.341 [0.122-4.382]	0.3, 1, 3	0.99	20.4
Flusilazole	Fungicides	85509-19-9	DTXSID3024235	0.085 [0.043-0.291]	0.03, 0.1, 0.3	1.6	13.2
Dieldrin	GABA Modulators	60-57-1	DTXSID9020453	1.035 [0.347-2.177]	0.3, 1, 3	0.7	11.6
Heptachlor Epoxide	GABA Modulators	1024-57-3	DTXSID1024126	1.737 [1.288-1.995]	0.3, 1, 3	1.5	19.5
Lindane	GABA Modulators	58-89-9	DTXSID2020686	0.799 [0.267-4.782]	0.3, 1, 3	0.5	ND
Fipronil	GABA Modulators	120068-37-3	DTXSID4034609	0.799 [0.158-3.089]	0.3, 1, 3	15.6	25.3
Cadmium	Metals	7790-78-5	DTXSID4040183	0.036 [0.036-0.043]	0.01, 0.03, 0.1	0.04	0.48
Lead Acetate	Metals	6080-56-4	DTXSID3031521	2.916 [1.828-4.382]	1, 3, 10	0.5	16.1
Tributyltin oxide	Metals	56-35-9	DTXSID9020166	0.005 [0.004-0.006]	0.001, 0.003, 0.01	0.02	0.03
Triethyltin bromide	Metals	2767-54-6	DTXSID9040712	0.022 [0.017-0.024]	0.003, 0.01, 0.03	0.07	0.06
Sodium Arsenite	Metals	7784-46-5	DTXSID5020104	0.092 [0.055-0.122]	0.03, 0.1, 0.3	1.0	1.1
Chlorpyrifos	Organophosphates	2921-88-2	DTXSID4020458	1.23 [0.072-1.828]	0.3, 1, 3	13.1	13.7
Chlorpyrifos oxon	Organophosphates	5598-15-2	DTXSID1038666	0.071 [0.039-0.133]	0.03, 0.1, 0.3	0.4	0.3
PBDE-47	Other	5436-43-1	DTXSID3030056	1.155 [0.302-2.262]	0.3, 1, 3	0.01	11.6
Permethrin	Other	52645-53-1	DTXSID8022292	0.871 [0.64-1.288]	0.3, 1, 3	5.7	9.2
Bis(2-ethylhexyl) phthalate	Other	117-81-7	DTXSID5020607	0.11 [0.055-0.224]	0.03, 0.1, 0.3	2.0	12.3
Methylchloroisothiazolinone	Other	26172-55-4	DTXSID9034286	0.436 [0.245-0.64]	0.1, 0.3, 1	2.0	4.61
Paraquat dichloride‡	Other	1910-42-5	DTXSID7024243	0.142 [0.102-0.205]	0.03, 0.1, 0.3	0.8	1.7

⁺Frank et al. (2018).

*From ToxCast; may differ from values reported in Frank et al. (2017).

‡CAS No. and DTXSID were incorrectly reported in Frank et al 2017 and 2018.

Treatments caused more metabolomic than transcriptomic changes

Differentially Expressed Genes (DEGs) and Significant Metabolites at Tested Doses

	No. DEGs			No. Metabolites		
Chemical	LOW	MID	HIGH	LOW	MID	HIGH
Tebuconazole	1	51	923	101	82	350
Flusilazole	-	-	-	133	72	49
Dieldrin	-	-	-	107	137	65
Heptachlor Epoxide	-	-	-	149	54	139
Lindane	-	-	-	557	202	86
Fipronil	96	125	208	139	75	135
Cadmium	-	-	8	114	432	62
Lead Acetate	96	221	895	148	295	392
Tributyltin oxide	-	13	103	148	157	117
Triethyltin bromide	-	-	-	585	399	244
Sodium Arsenate	-	-	9	48	81	415
Chlorpyrifos	-	1	-	65	75	48
Chlorpyrifos oxon	-	-	-	44	79	68
PBDE-47	-	-	-	41	96	125
Permethrin	-	-	32	78	285	125
Bis(2-ethylhexyl) phthalate	-	-	-	243	137	113
Methylchloroisothiazolinone	-	-	249	215	384	194
Paraquat	-	-	1	57	93	114

Many fewer DEGs observed here. Therefore, focus on compounds with concentration-dependent changes in DEGs.

Top Diseases and Functions Associated With Functional Alterations in Network Development

Tebuconazole

- Cell Death and Survival
- Neurological Disease
- Organismal Injury and Abnormalities
- Cellular Development
- Nervous System Development and Function

Lead Acetate

- Cell Morphology,
- Cellular Assembly and Organization
- Molecular Transport
- Cell-To-Cell Signaling and Interaction
- Nervous System Development and Function
- Neurological Disease
- Psychological Disorders

Tributyltin

- Amino Acid Metabolism
- Cell-To-Cell Signaling and Interaction
- Cell Death and Survival
- Cellular Growth and Proliferation
- Neurological Disease
- Nervous System Development and Function

Fipronil

- Cell Morphology
- Cell Development
- Tissue Morphology
- Neurological Disease
- Nervous System Development and Function,

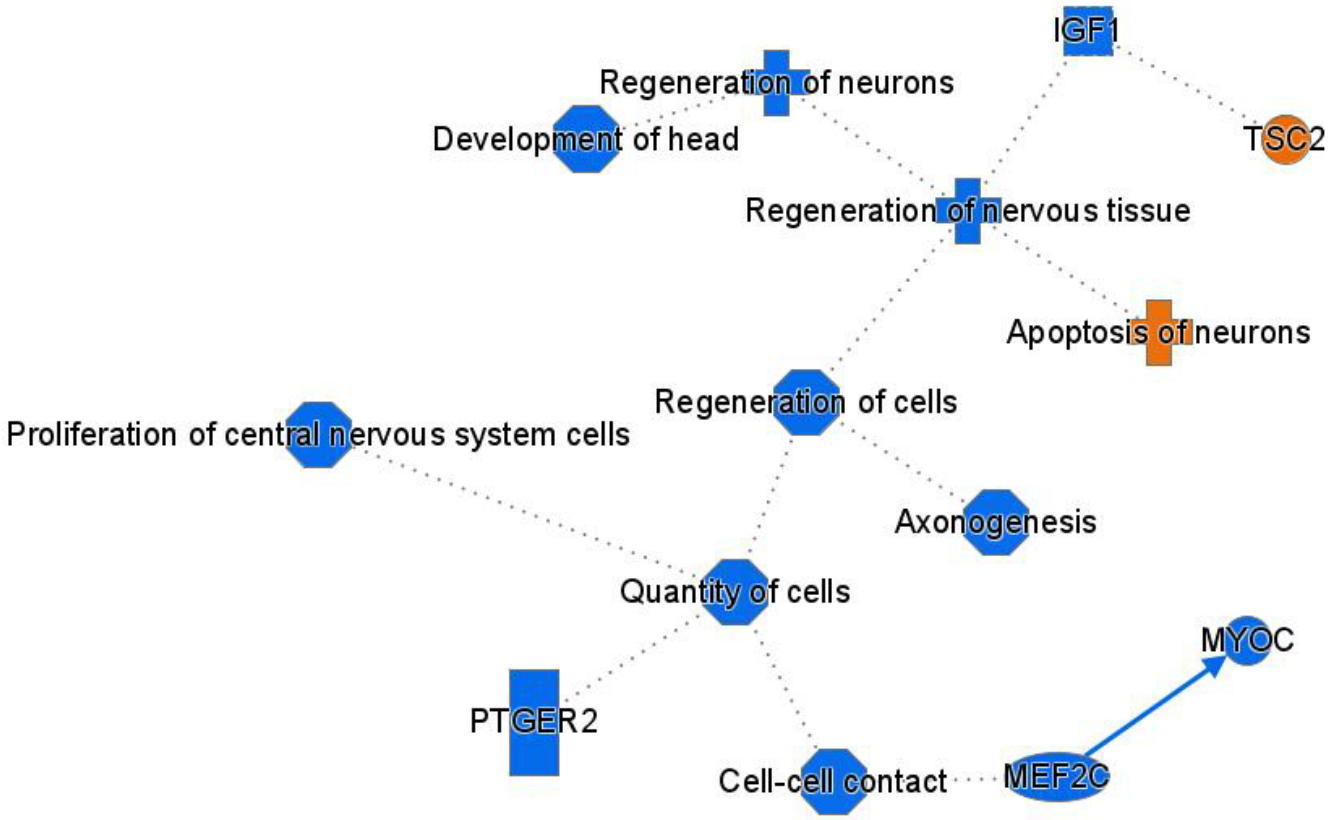
Top Upstream Regulators

Cytosine Arabinoside (CA)	5-Fluorouracil (5FU)	Domoic acid (DA)	Cypermethrin (CM)	Deltamethrin (DM)	Haloperidol (HP)	Lead Acetate	Tributyltin Chloride	Fipronil	Tebuconazole
CREB1↑	SOX2↓	LOC102724788/	ARX	ATXN1	CREB1	SOX2↓	MAPT	SOX2↑	TARDBP↑
SOX2↓	TCF7L2↓	PRODH	LOC102724788/	GLS	CA9	KDM1A	PSEN1	BDNF	PROM1
ZBTB17	PRMT1↓	SLC1A2	PRODH	HPRT1	CPT1B	PPP3CA	APP	CHMP2B	SH3TC2↑
S100A8↑	PPP3CA	ELAVL4	SLC1A2	ELAVL4		inosine↓	HTT	CREB1	LMNB1
	NOTCH1	GLS	KLF9			MYOC↓	BDNF↑	HTT	SIRT2↑
			SLC23A2			MAPT	MKNK1↑	GRN	SOX2↑
						CX3CL1	ATN1	SERPINF1	NR1H3
						HTT	FMR1↓	CSF1	NR1H2
						APP↓	GRN		CYP27A1↑
							CYP27A1		

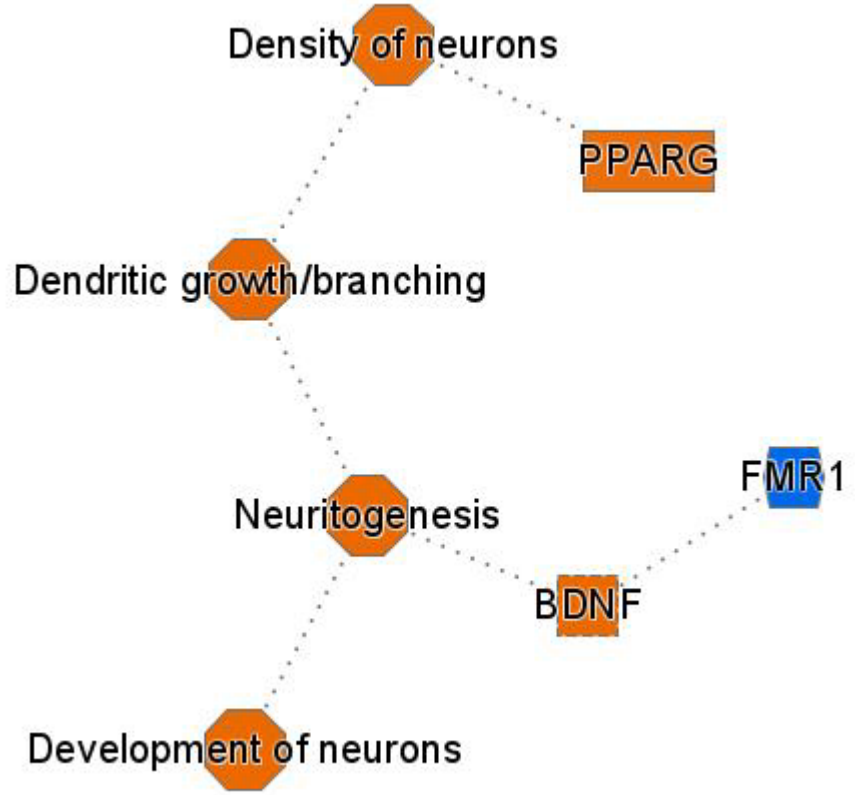
Our analysis indicates that there are a number of common upstream regulators across the active chemicals. However, there is also considerable heterogeneity in the identified upstream regulators.

Analysis could inform AOPs

Lead- Mid dose



Tributyltin- Mid dose



Conclusions

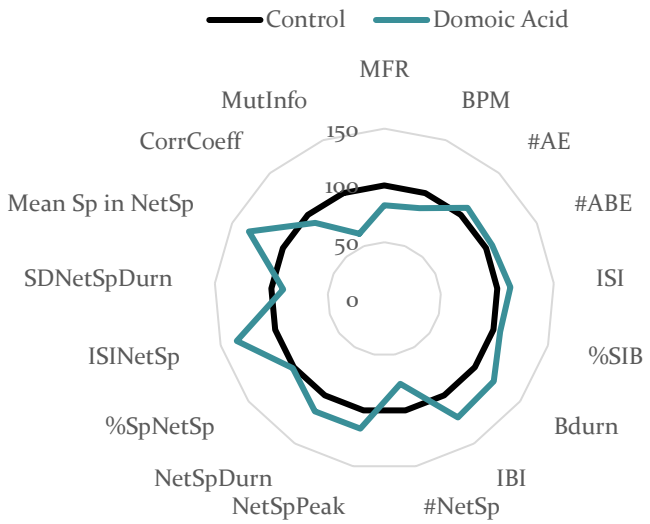
- To date, there are few AOPs focused on DNT Adverse Outcomes
  - Especially if thyroid-related AOPs are considered (excluded)
- The Omics approaches identify common diseases, molecular functions, upstream regulators and canonical pathways associated with positive responses in the DNT NAMs
  - Data for more chemicals are needed to confirm this
  - If common regulators and pathways are identified, this might result in novel DNT NAMs
- Omics approaches also identify unique responses to each chemical at multiple levels of organization.
- Using Omics approaches and NAMs data, putative AOPs can be identified
  - These putative AOPs need development, but provide a link between DNT NAMs data, key events and adverse outcomes related to human disease

Thank you!
Questions?

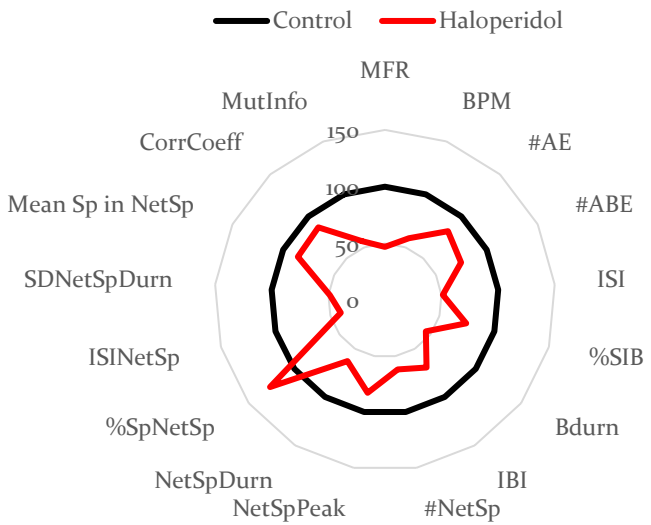
EPA ORD Colleagues:

- Kathleen Wallace
- Theresa Freudenrich
- Brian Chorley
- David Gallegos
- Carmen Marable
- Chris Frank
- Matthew Henderson
- Susan Hester
- Roland Seim

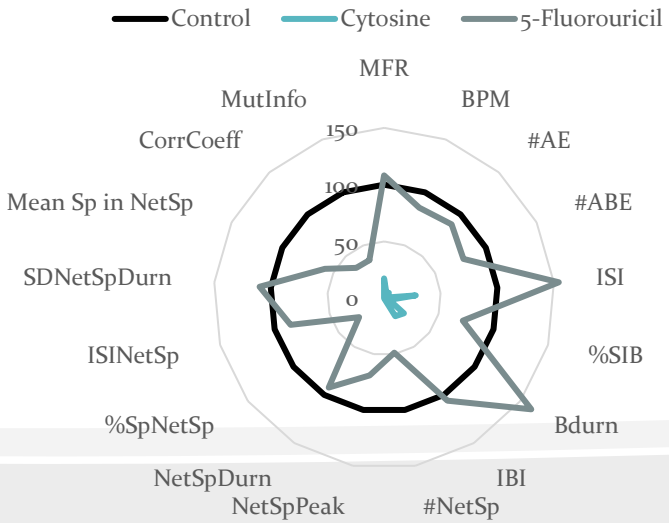
Domoic Acid



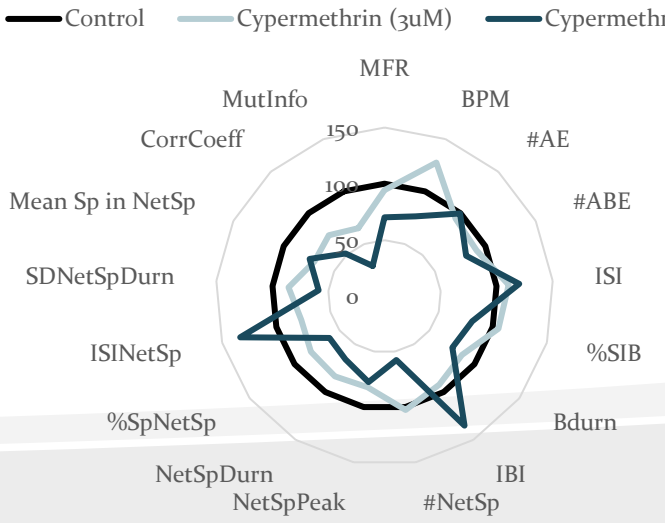
Haloperidol



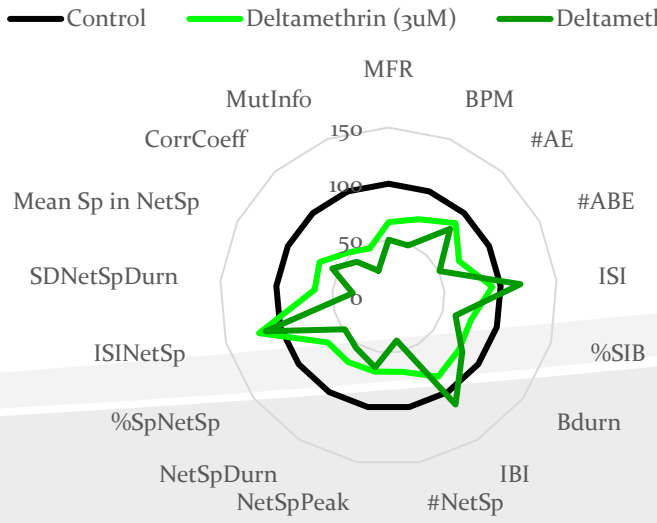
DNA Synthesis Inhibitors



Cypermethrin



Deltamethrin



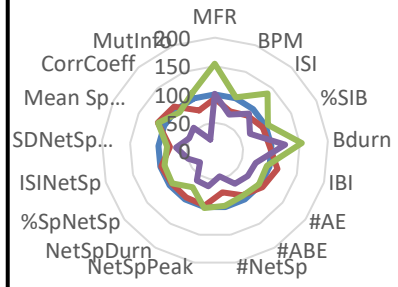
Marable et al., Toxicol Sci. 2022 Feb 28;186(1):118-133. doi: 10.1093/toxsci/kfab151

Functional alterations in the MEA NFA

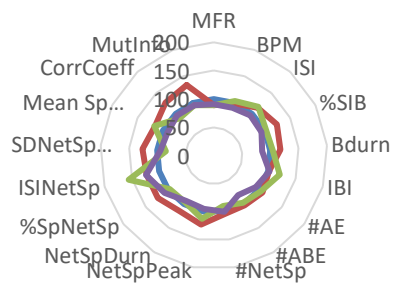
Fungicides
GABA-A Receptor
Metals
Parent/metabolite

EPA

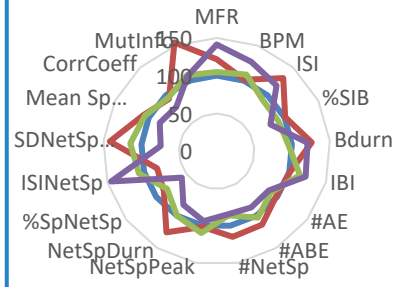
Tebuconazole



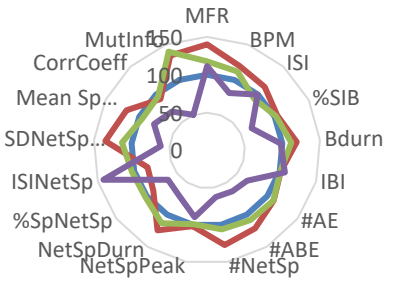
Flusilazole



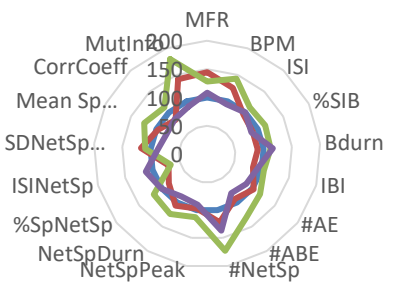
Cadmium



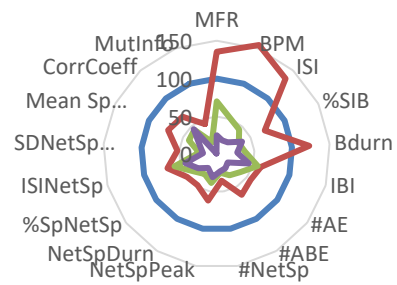
Triethyltin bromide



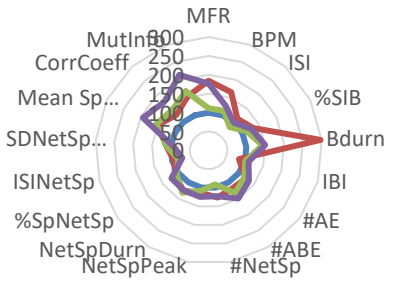
Tributyltin Oxide



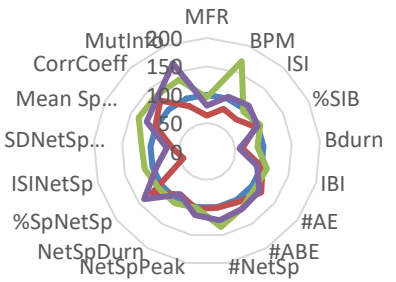
Lead Acetate



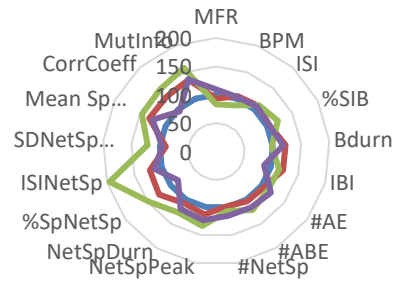
Heptachlor Epoxide



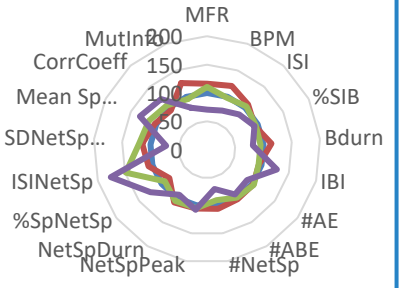
Lindane



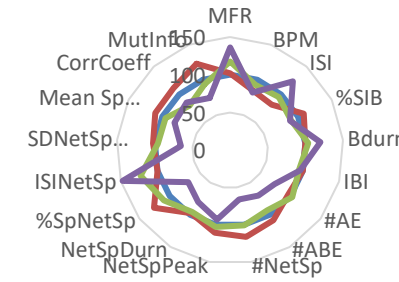
Bis(2-ethylhexyl) phthalate



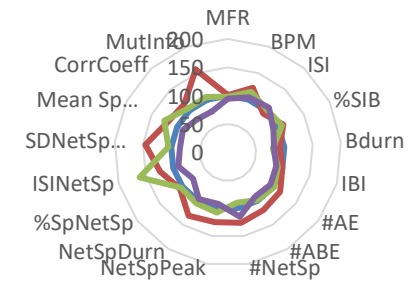
Sodium Arsenite



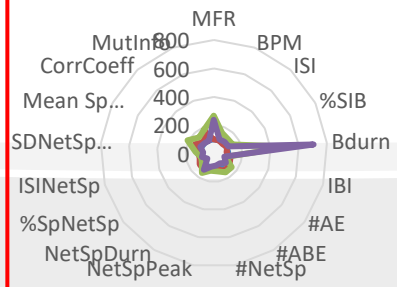
PBDE-47



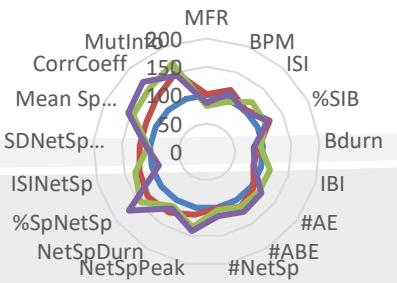
Methylchloroisothiazolinone



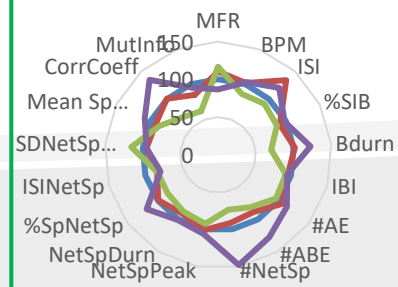
Dieldrin



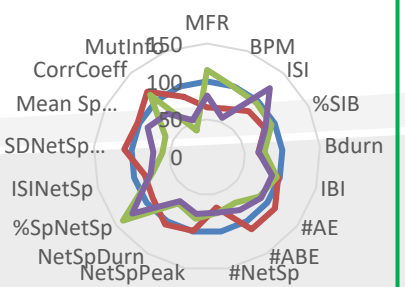
Fipronil



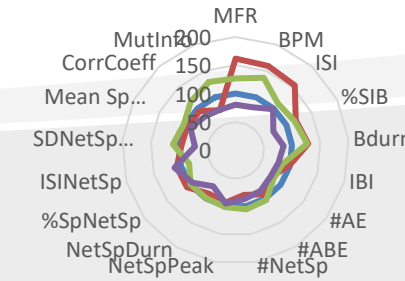
Chlorpyrifos Oxon



Chlorpyrifos



Permethrin



Paraquat