

Developmental Neurotoxicity Assessment Using In Vitro Assays

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ODS (Office of Data Science)

OPO (Office of Program Operations)

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MTB (Mechanistic Toxicology Branch)

STB (Systems Toxicology Branch)

Division of Intramural Research (DIR)

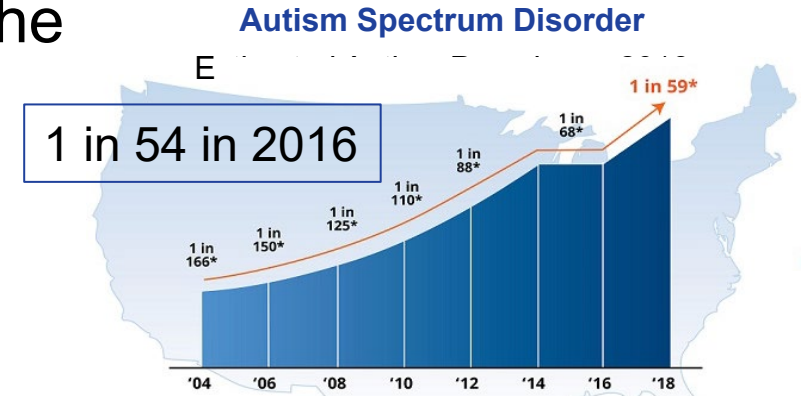
NL (Neurobiology Laboratory)

*Now Neurocrine Biosciences

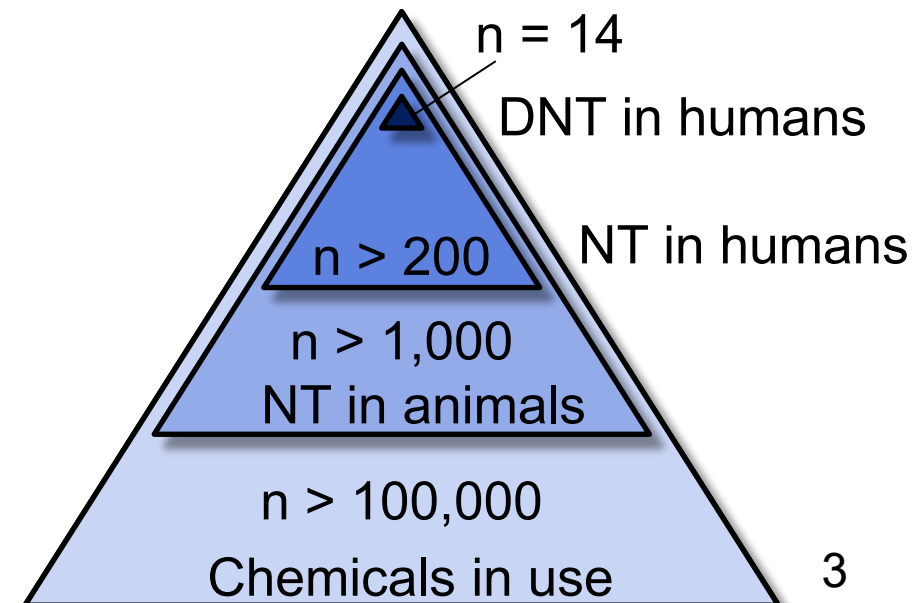


Importance of Assessing Developmental Neurotoxicity (DNT)

- Increase in prevalence of developmental disorders in the US and globally
 - WHO: 1 in 6 kids diagnosed at birth
- *In vivo* DNT Guideline studies primary method of evaluation
 - Require an *a priori* trigger to be run
 - Time & resource intensive
 - Relevance of animal studies for human translation have been questioned
- Compounds with unknown DNT and NT potential remain untested



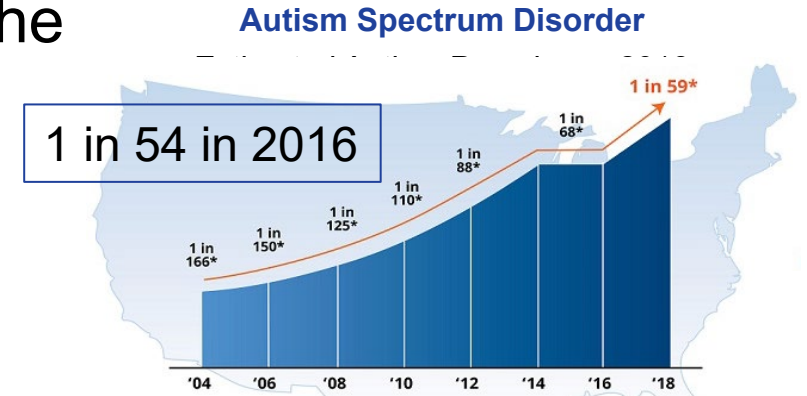
* Centers for Disease Control and Prevention (CDC) prevalence estimates are for 4 years prior to the report date (e.g. 2018 figures are from 2014)
Source: autismspeaks.org, "CDC increases estimate of autism's prevalence by 15 percent, to 1 in 59 children"



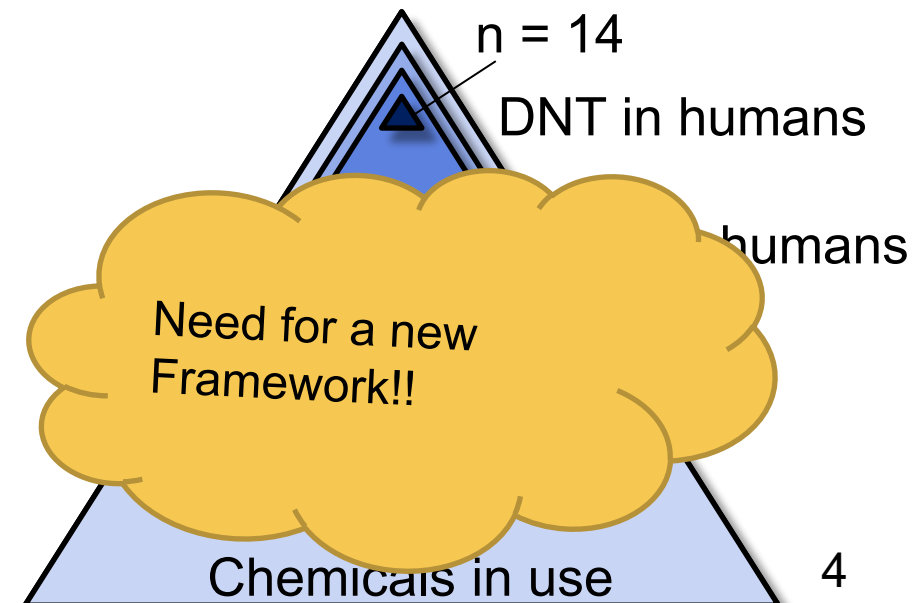


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Regulatory Focus on Developing New Frameworks

Perspectives | Brief Communication

Project TENDR: Targeting Environmental Neuro-Developmental Risks. The TENDR Consensus Statement

<http://dx.doi.org/10.1289/EHP358>

Toxicology and Applied Pharmacology 354 (2018) 3-6



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



Consensus statement on the need for innovation, transition and implementation of developmental neurotoxicity (DNT) testing for regulatory purposes



Ellen Fritsche^a, Philippe Grandjean^b, Kevin M. Crofton^c, Michael Aschner^d, Alan Goldberg^{e,w}, Tuula Heinonen^f, Ellen V.S. Hessel^g, Helena T. Hogberg^h, Susanne Hougaard Bennekouⁱ, Pamela J. Lein^j, Marcel Leist^k, William R. Mundy^l, Martin Paparella^m, Aldert H. Piersmaⁿ, Magdalini Sachana^o, Gabriele Schmuck^p, Roland Solecki^q, Andrea Terron^r, Florianne Monnet-Tschudi^s, Martin F. Wilks^t, Hilda Witters^u, Marie-Gabrielle Zurich^s, Anna Bal-Price^{v,*}



FIFRA Scientific Advisory Panel Meeting Minutes and Final Report No. 2020-02

Peer Review of the Use of New Approach Methodologies (NAMs) to Derive Extrapolation Factors and Evaluate Developmental Neurotoxicity for Human Health Risk Assessment

September 15-18, 2020

FIFRA Scientific Advisory Panel Meeting

TOXICOLOGICAL SCIENCES, 167(1), 2019, 45-57

doi: 10.1093/toxsci/kfy211

Advance Access Publication Date: November 23, 2018

Forum

SOT | Society of Toxicology
www.toxsci.oxfordjournals.org

OXFORD

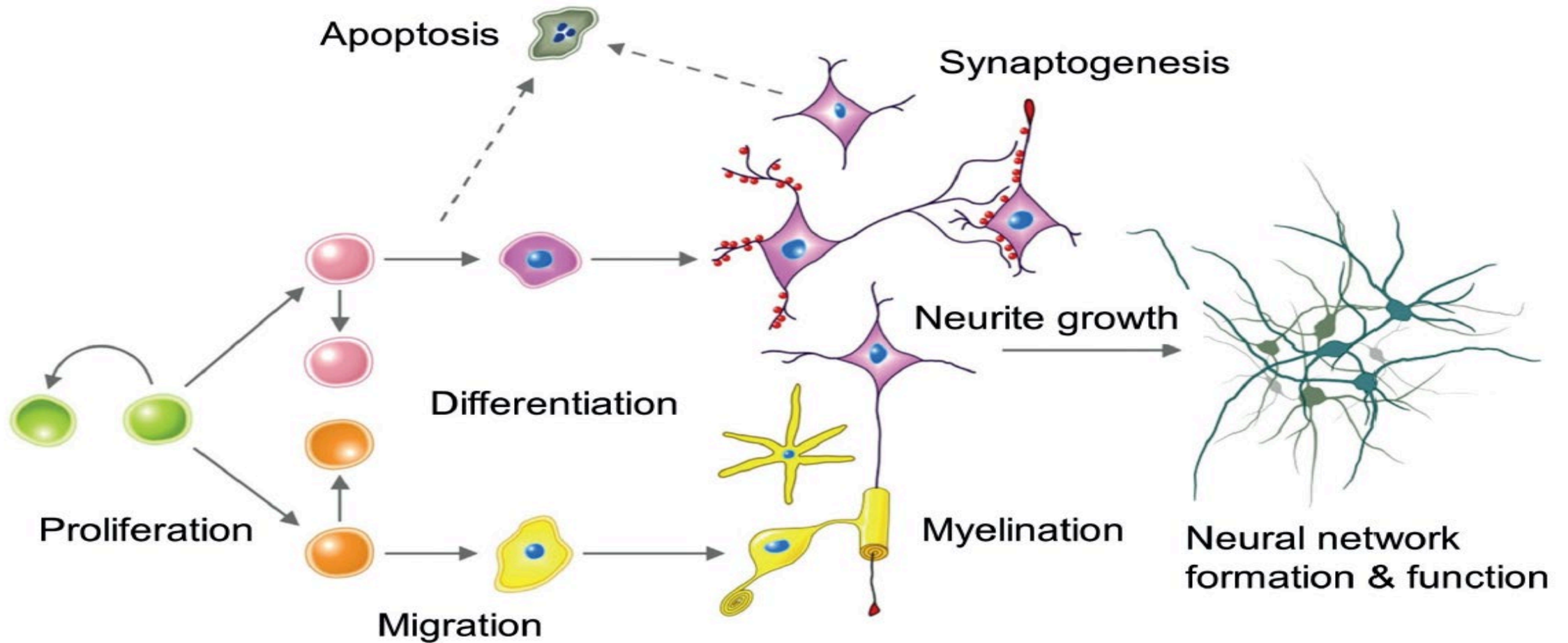
FORUM

International Regulatory and Scientific Effort for Improved Developmental Neurotoxicity Testing

Magdalini Sachana,^{*,1} Anna Bal-Price,[†] Kevin M. Crofton,[‡] Susanne H. Bennekou,[§] Timothy J. Shafer,^{||} Mamta Behl,^{||} and Andrea Terron^{|||}



In vitro Assays to Model Neurodevelopmental Key Events





Battery of Tests to Cover the Key Events

Phase I: Test development

Phase II: Performance and replicability

Phase III: Screening

Readiness/ Test method	Phase I	Phase II	Phase III	Overall readiness
UKN1	A	B	B	B+
NPC1	A	A	A	A
NPC2	A	A	A	A
NPC3	A	A	B	A-
NPC4	A	B	C	B
NPC5	A	A	B	A-
NPC6	A	B	B	B+
UKN2 (cMINC)	A	B	A	A-
MESn	C	D	D	D+
UKN4 (NeuriTox)	A	A	A	A
UKN5 (PeriTox)	A	B	A	A-
NSR	C	D	D	D+
SYN	B	B	B	B
Nnff	B	A	B	B+
3Dr	A	A	A	A
3Dh	B	C	C	C+
ZFE	B	B	A	B+

Integrated Approach to Testing and Assessment (IATA) for DNT
 1st Workshop Report* Bal-Price et al., 2018, ALTEX

Recommendation on Test Readiness Criteria for New Approach Methods in Toxicology: Exemplified for Developmental Neurotoxicity

Anna Bal-Price¹, Helena T. Hogberg², Kevin M. Crofton³, Mardas Daneshian⁴, Rex E. FitzGerald⁵, Ellen Fritsche⁶, Tuula Heinonen⁷, Susanne Hougaard Bennekou⁸, Stefanie Klima⁹, Aldert H. Piersma¹⁰, Magdalini Sachana¹¹, Timothy J. Shafer³, Andrea Terron¹², Florianne Monnet-Tschudi^{5,13}, Barbara Viviani¹⁴, Tanja Waldmann⁹, Remco H. S. Westerink¹⁵, Martin F. Wilks⁵, Hilda Witters¹⁶, Marie-Gabrielle Zurich^{5,13} and Marcel Leist^{4,9}

Cellular system	NEP diff.	Neuro-spheres	ReNcell	Neural crest migration	hESC / hiPS based diff.	CNS neurons	3D human cell culture	3D rat cell culture	2D murine cell culture	PNS neurons	Zebra fish
Name of assay	NEP diff.	NPC 1-6	ReNcell	UKN2 (cMINC)	UKN1	UKN4 (NeuriTox)	3Dh	3Dr	2Dm	UKN5 (PeriTox)	ZFE



Battery of Tests to Cover the Key Events

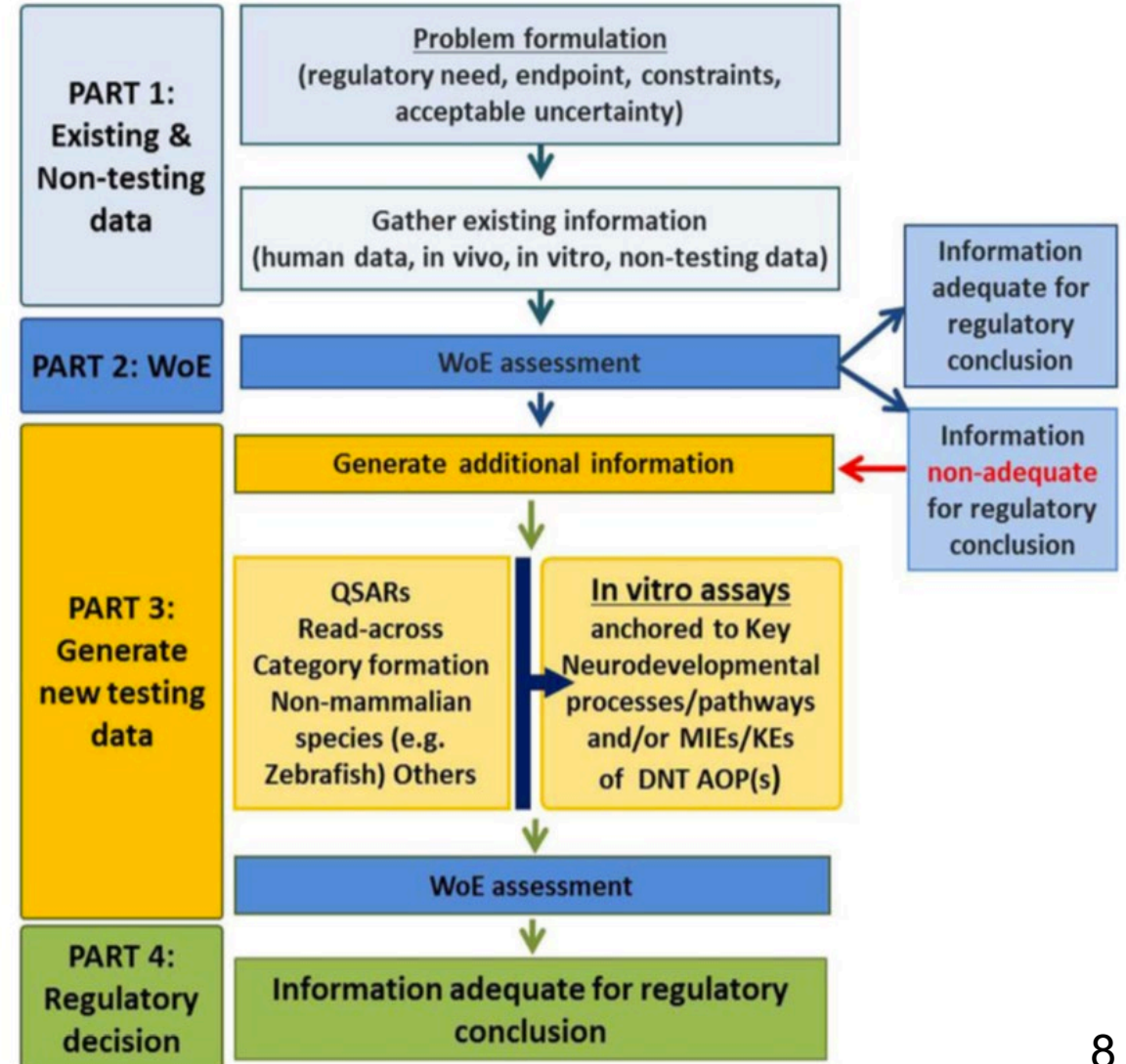
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Integrated Approach to Testing and Assessment (IATA) for DNT





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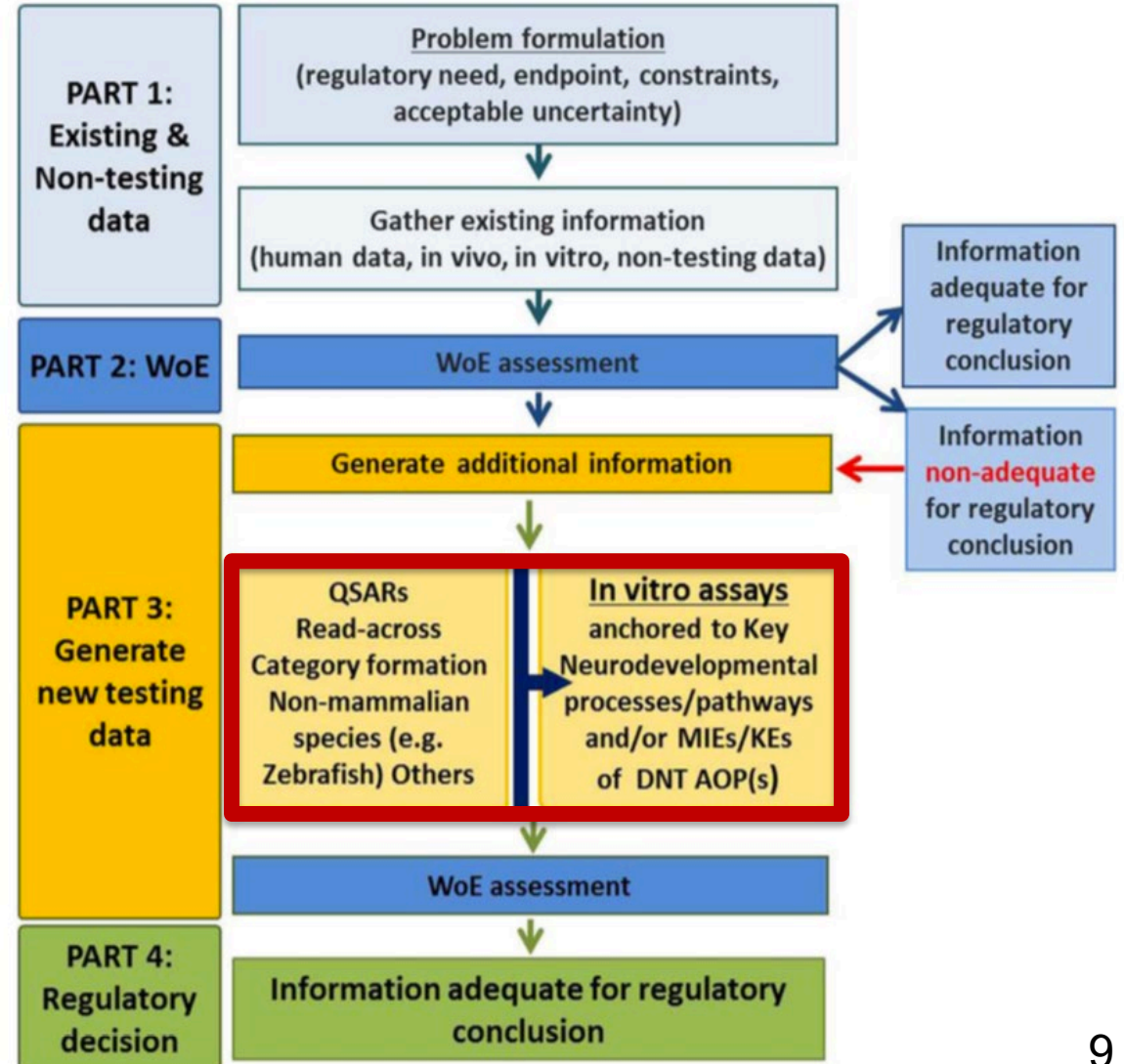
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Integrated Approach to Testing and Assessment (IATA) for DNT





Expert Group on DNT



Guidance Document

To inform on the testing battery, its usage and interpretation. Case studies exemplifying different regulatory needs.



Expert Group on DNT



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Case study led by DNTP



Title: Organophosphorus flame retardants, a case study on the use of IATA for DNT to prioritize a class of compounds

Authors: Helena Hogberg, Jui-Hua Hsieh, Xiaoqing Chang, Nisha Sipes, Tim Shafer, Mamta Behl



Regulatory Support and Application



Expert Group on DNT



Guidance Document

FIFRA review
NAMs for DNT



2021- EPA Uses
NAMs for DNT to
support waiving a
guideline DNT study

To inform on the testing battery, its usage and interpretation. Case studies exemplifying different regulatory needs.

Case study led by DNTP



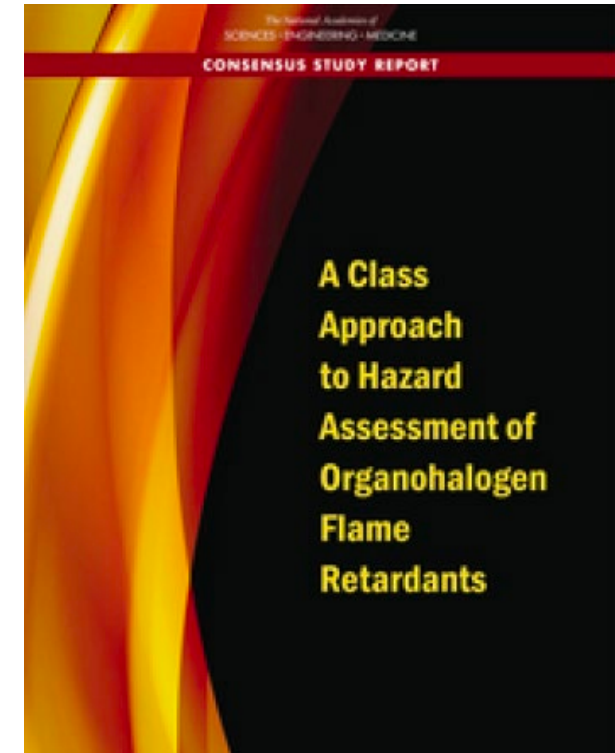
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Need for class evaluation of Flame Retardants

- Consumer Product Safety Commission (CPSC) petitioned to initiate regulatory action to ban certain flame retardant products
- CPSC must first conduct hazard assessment on classes of flame retardants
- Moving away from traditional chemical-by-chemical approach to classes
 - Not practical to test every compound in a traditional in vivo study
 - Chemicals on which data are insufficient are typically treated as not hazardous
 - Untested chemicals often substituted for hazardous chemicals
 - Cumulative exposure and risk are often ignored
- Recognizes challenging to evaluate chemical groups





Class Evaluation of Flame Retardants (NAS 2019)

Organophosphate Flame Retardants are one class in NAS report

- 20-50 compounds in class including commercial and isomeric mixtures
- Continual rise and increase in human exposure
- Aliphatic halogenated OPFRs and aromatic non-halogenated
- Cannot test our way through all combinations using traditional animal guideline studies
- Need strategy to prioritize compounds for further in-depth hazard characterization



CAS	Chemical Name	Chemical.ID	Structure
Representative Brominated FRs (BFRs)			
5436-43-1	2,2'4,4'-Tetrabromodiphenyl ether	BDE-47	
79-94-7	3,3',5,5'-Tetrabromobisphenol A	TBBPA	
Organophosphorous FRs (OPFRs)- aliphatic, halogenated			
13674-87-8	Tris(1,3-dichloro-2-propyl)phosphate	TDCIPP	
115-96-8	Tris(2-chloroethyl) phosphate	TCEP	
Organophosphorous FRs (OPFRs)- Aromatic			
115-86-6	Triphenyl phosphate	TPHP	
68937-41-7	Phenol, isopropylated, phosphate (3:1)	IPP*	
1241-94-7	2-Ethylhexyl diphenyl phosphate	EHDP*	
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Phased-out (BDE)

Extensively used (and studied)

*representative isomer in mixture is shown as structure



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Phased-out (BDE)

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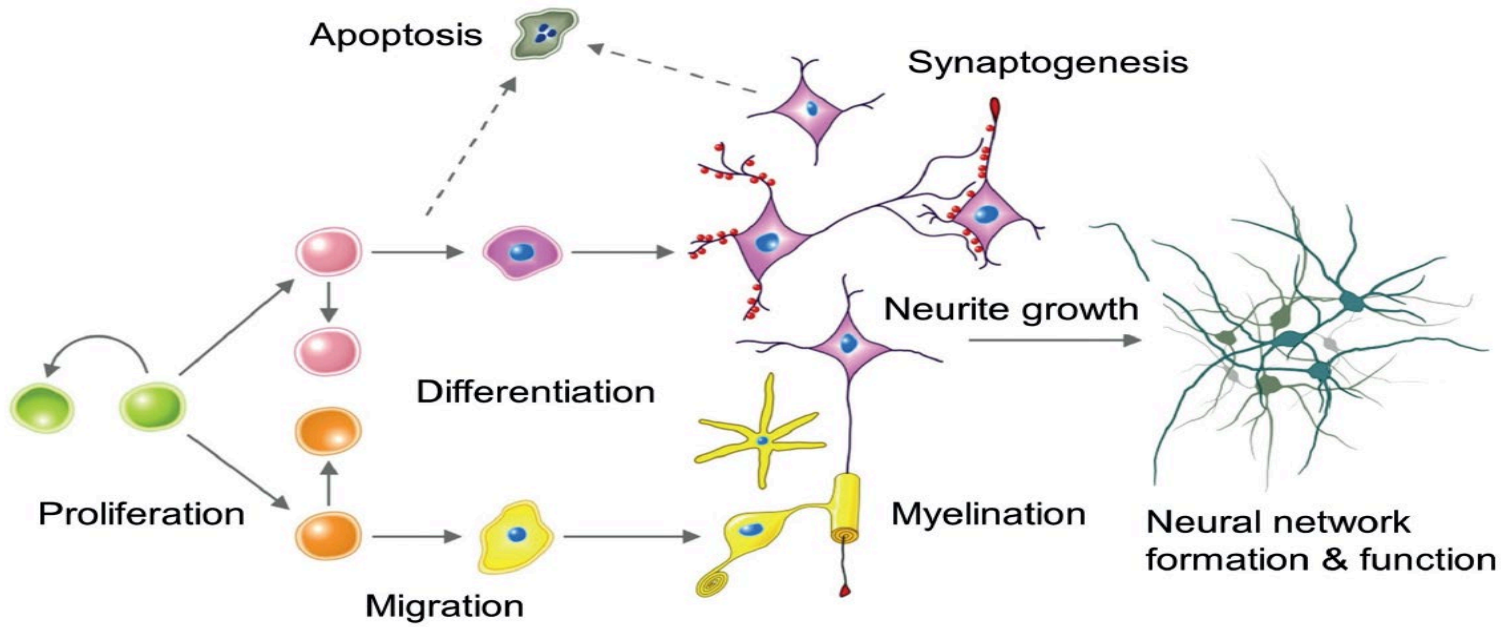
Aliphatic organohalogens

Aromatic phosphates (non-halogenated)

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Battery of Tests Covers Key Neurodevelopmental Events

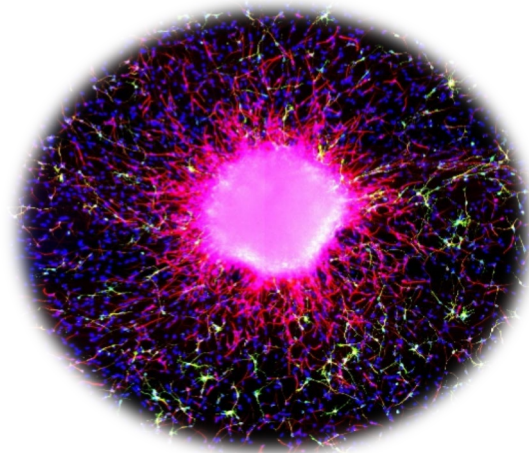


Cell lines, primary cells, stem cells, mainly human derived

2-D assays



3D- Neurospheres



Zebrafish





Datasets from the literature were re-evaluated using the BMC approach to compare assays in a unified way.

See DNT-DIVER

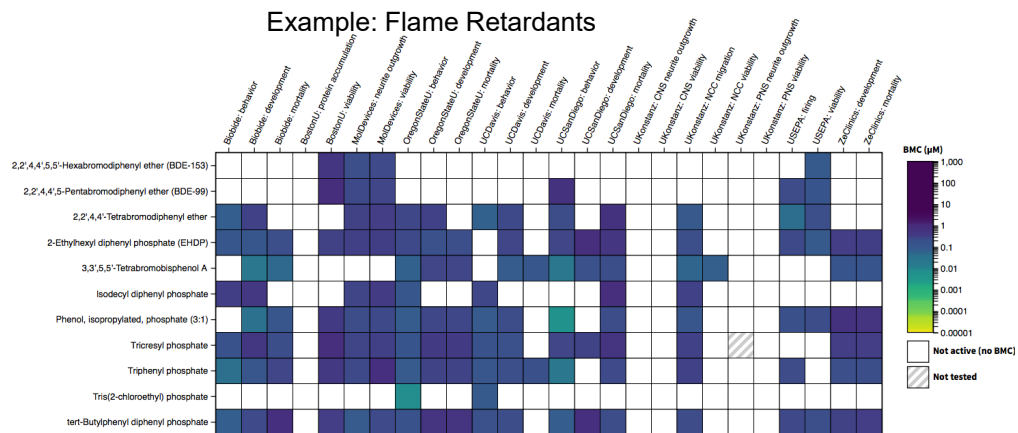
<https://sandbox.ntp.niehs.nih.gov/neurotox/>

Assay	Model	References
Proliferation		
- proliferation@IUF	Human 3D neurosphere	(Klose et al. 2021)
- proliferation@USEPA	Human hNP1	(Behl et al. 2015)
Oligo differentiation		
- oligodendrocyte differentiation@IUF	Human 3D neurosphere	(Klose et al. 2021)
Migration		
- NCC migration@UKonstanz	Human crest cells	(Nyffeler et al. 2017)
- neuronal migration@IUF*	Human 3D neurosphere	(Klose et al. 2021)
- oligo migration@IUF*	Human 3D neurosphere	(Klose et al. 2021)
Neurite outgrowth		
- neurite outgrowth@USEPA	Rat primary cortical	(Behl et al. 2015)
- neurite outgrowth@MolDevices	Human iPSC-derived	(Ryan et al. 2016)
- neurite outgrowth@USEPA	Human hN2	(Behl et al. 2015)
- CNS neurite outgrowth@UKonstanz	Human LUHMES	(Delp et al. 2018)
- PNS neurite outgrowth@UKonstanz	Human ESC-derived	(Delp et al. 2018)
- neurite outgrowth@IUF*	Human 3D neurosphere	(Klose et al. 2021)
Firing/Network formation		
- acute neuronal firing@USEPA	Rat primary cortical	(Behl et al. 2015)
- network formation@USEPA	Rat primary cortical	(Frank et al. 2017)
Behavior		
- behavior@Biobide	Zebrafish	(Quevedo et al. 2019)
- behavior@OregoneStateU	Zebrafish	(Hagstrom et al. 2019)
- behavior@UCDavis	Zebrafish	(Dach et al. 2019)



DNT- Data Integration and Visualization Enabling Resource

What can you do in DNT-DIVER?

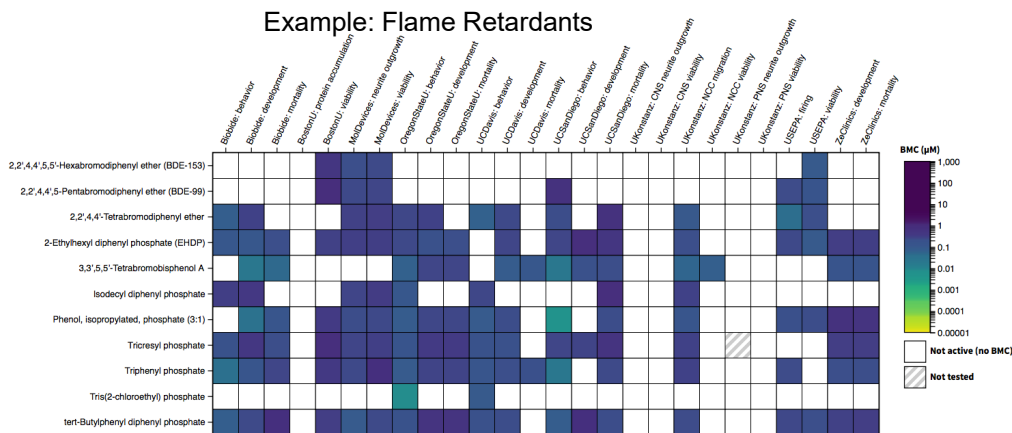


Compare activity of compounds/classes across multiple assays

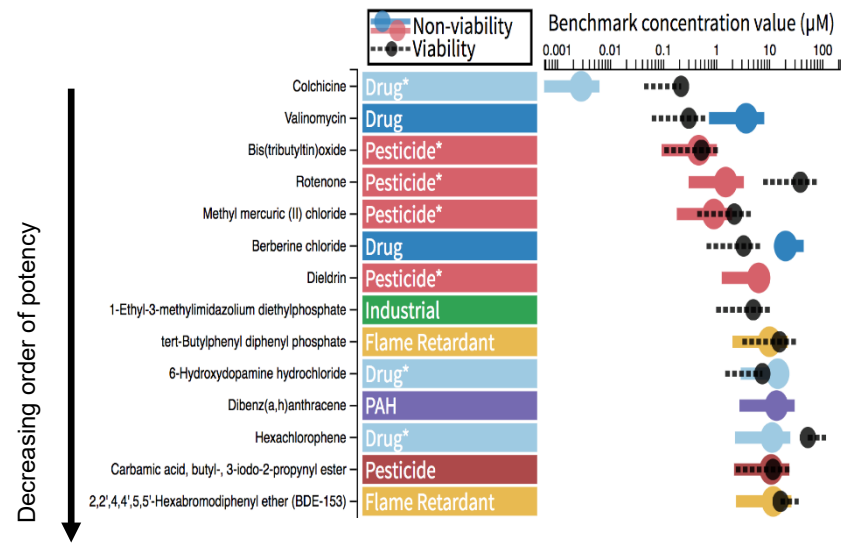


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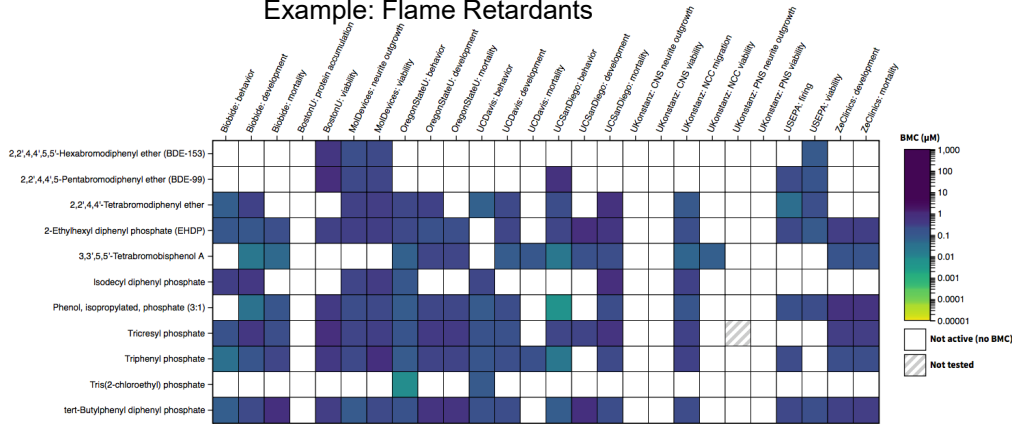
Compare activity of compounds within an assay



DNT- Data Integration and Visualization Enabling Resource

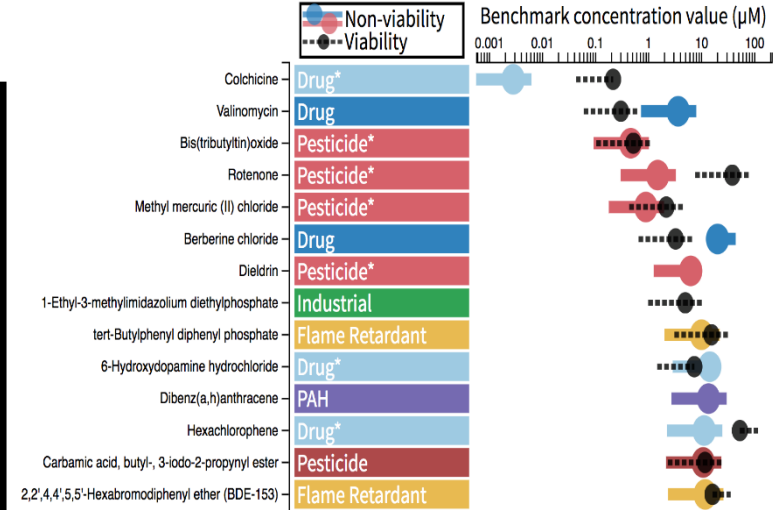
What can you do in DNT-DIVER?

Example: Flame Retardants

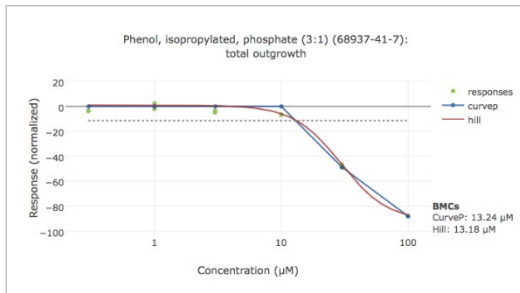


Compare activity of compounds/classes across multiple assays

Decreasing order of potency



Compare activity of compounds within an assay



Individual dose-response curves

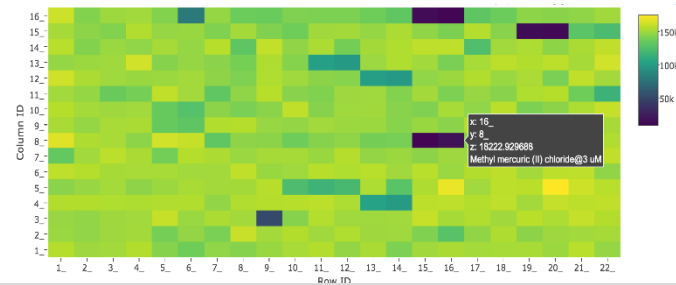
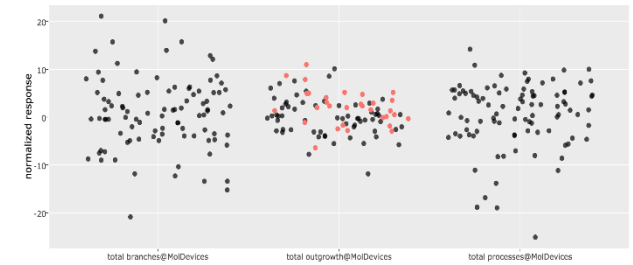


Plate and well level information

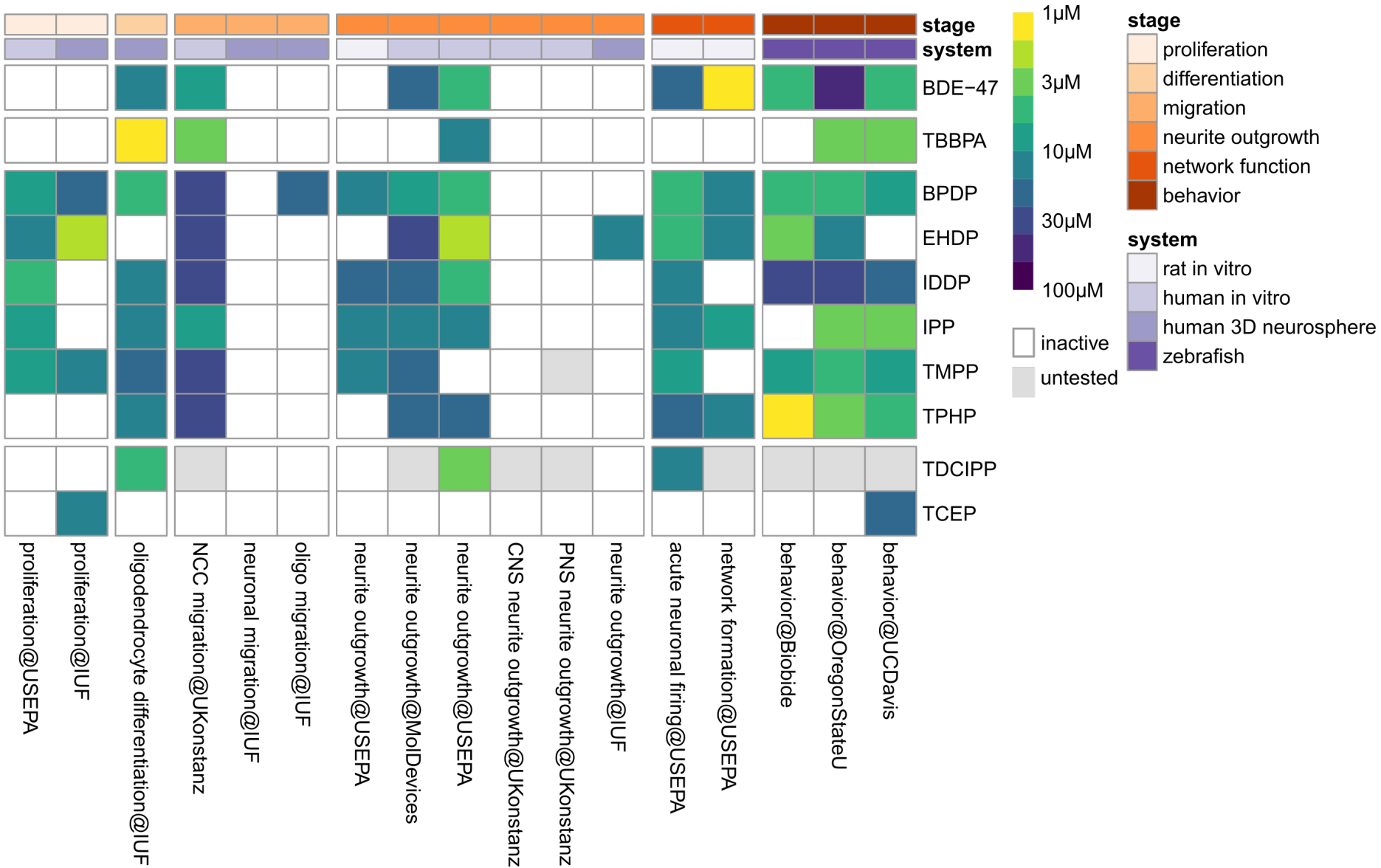


Control variability in assay

<https://sandbox.ntp.niehs.nih.gov/neurotox/>

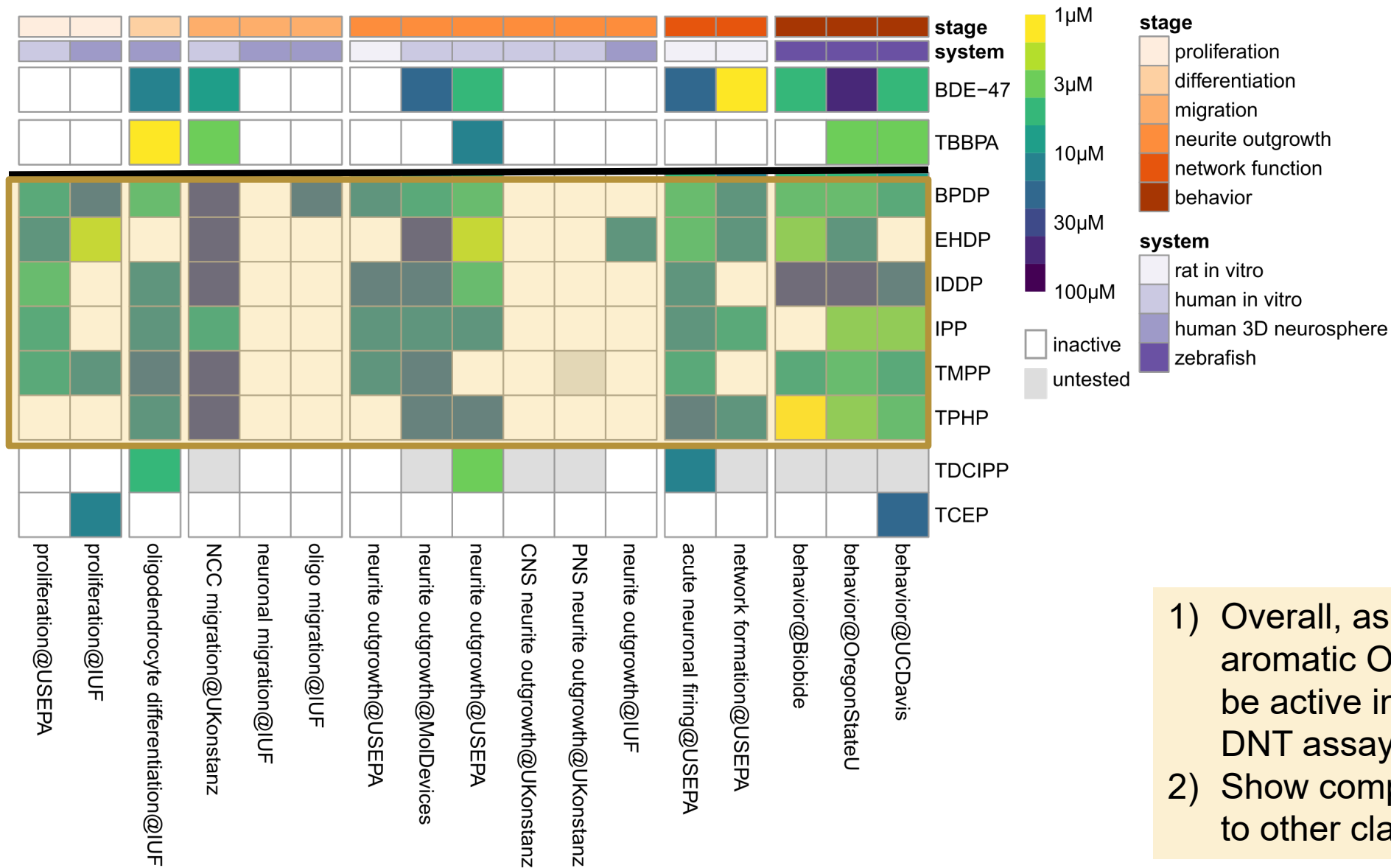


Summary of Findings





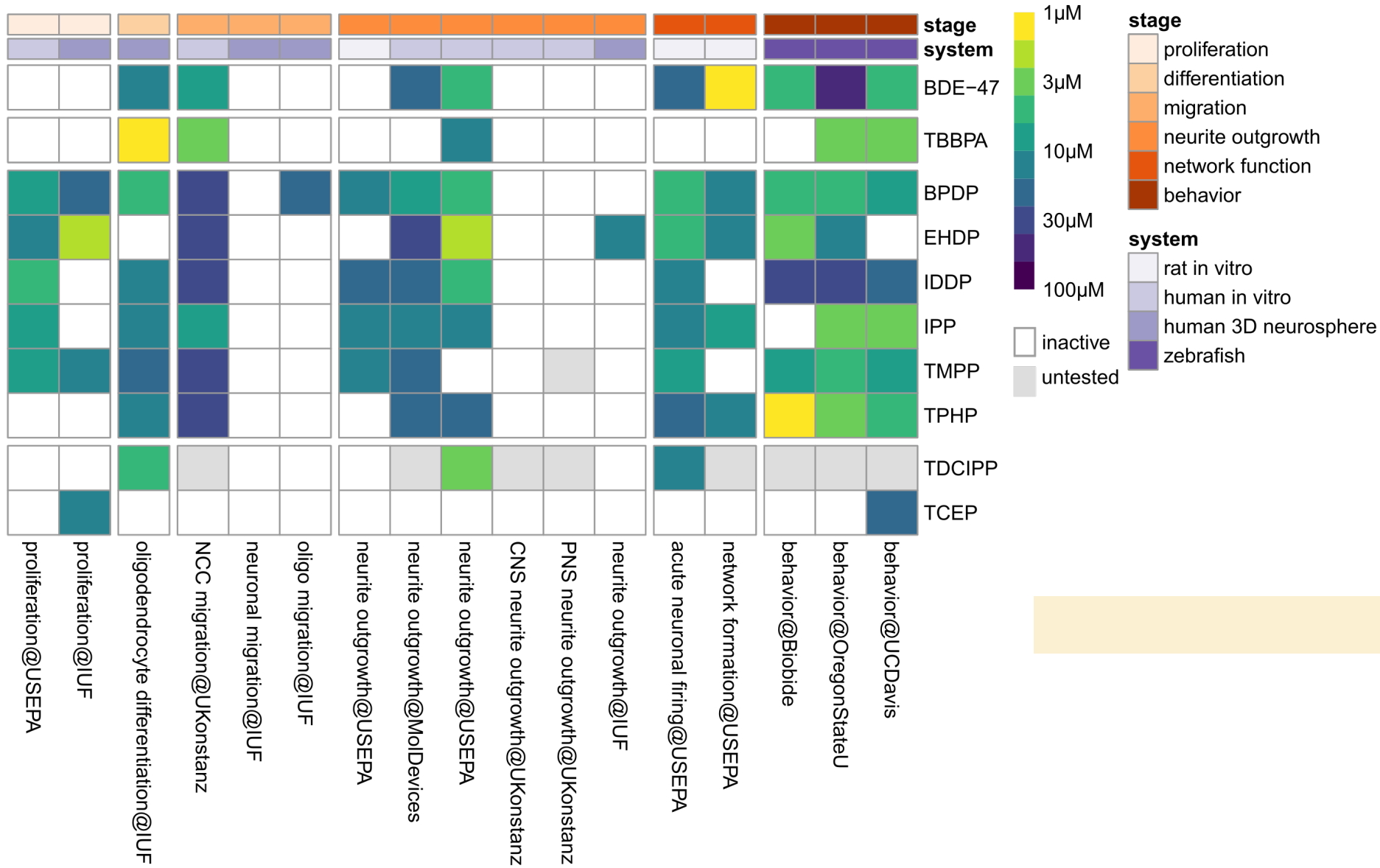
Summary of Findings



- 1) Overall, as a class the aromatic OPFRs appear to be active in a variety of DNT assays
- 2) Show comparable activity to other classes

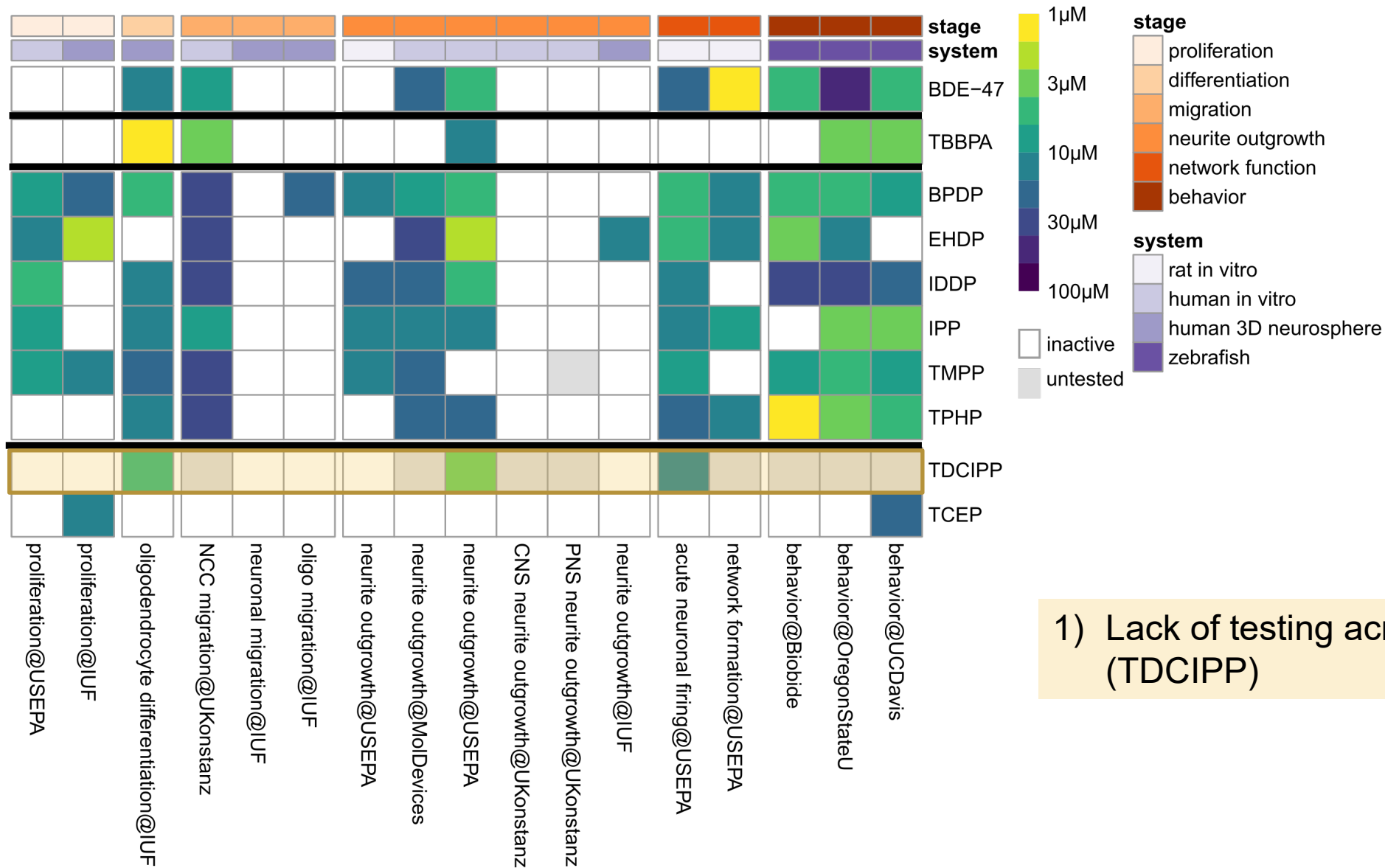


Sources of Uncertainty





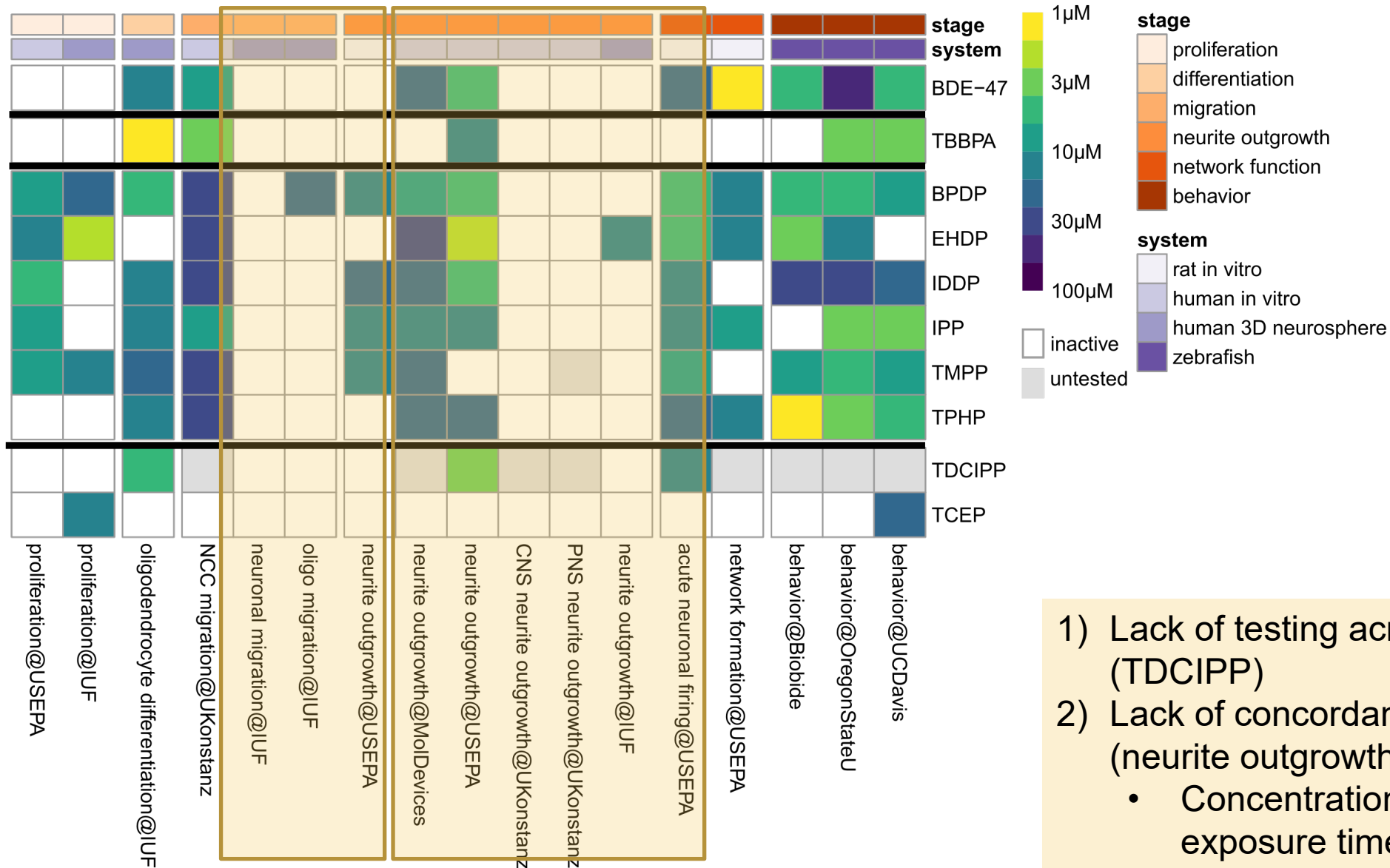
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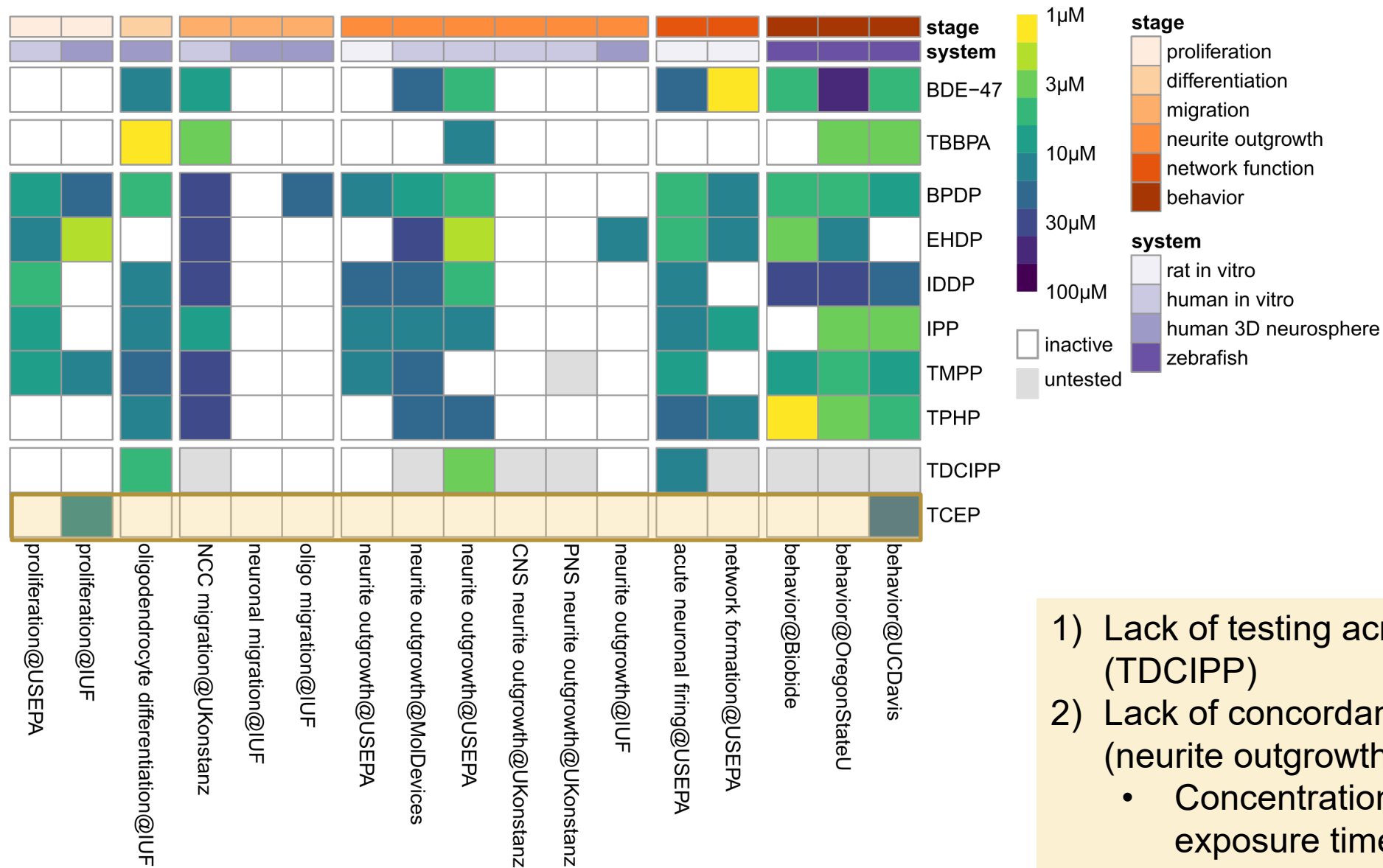
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- 2) Lack of concordance within assays (neurite outgrowth, migration)
 - Concentrations, models, exposure time



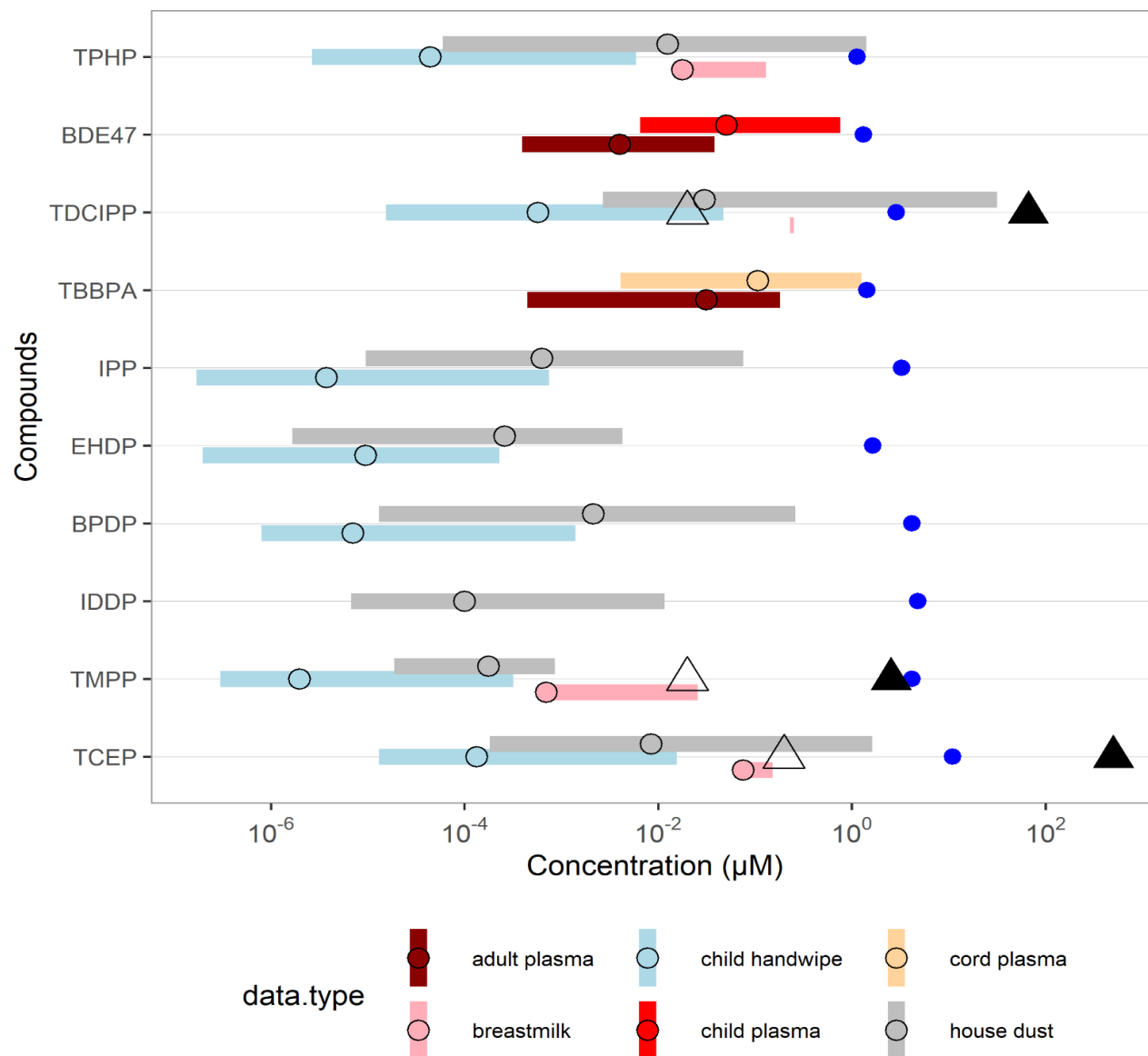
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- 3) Number of hits (TCEP)



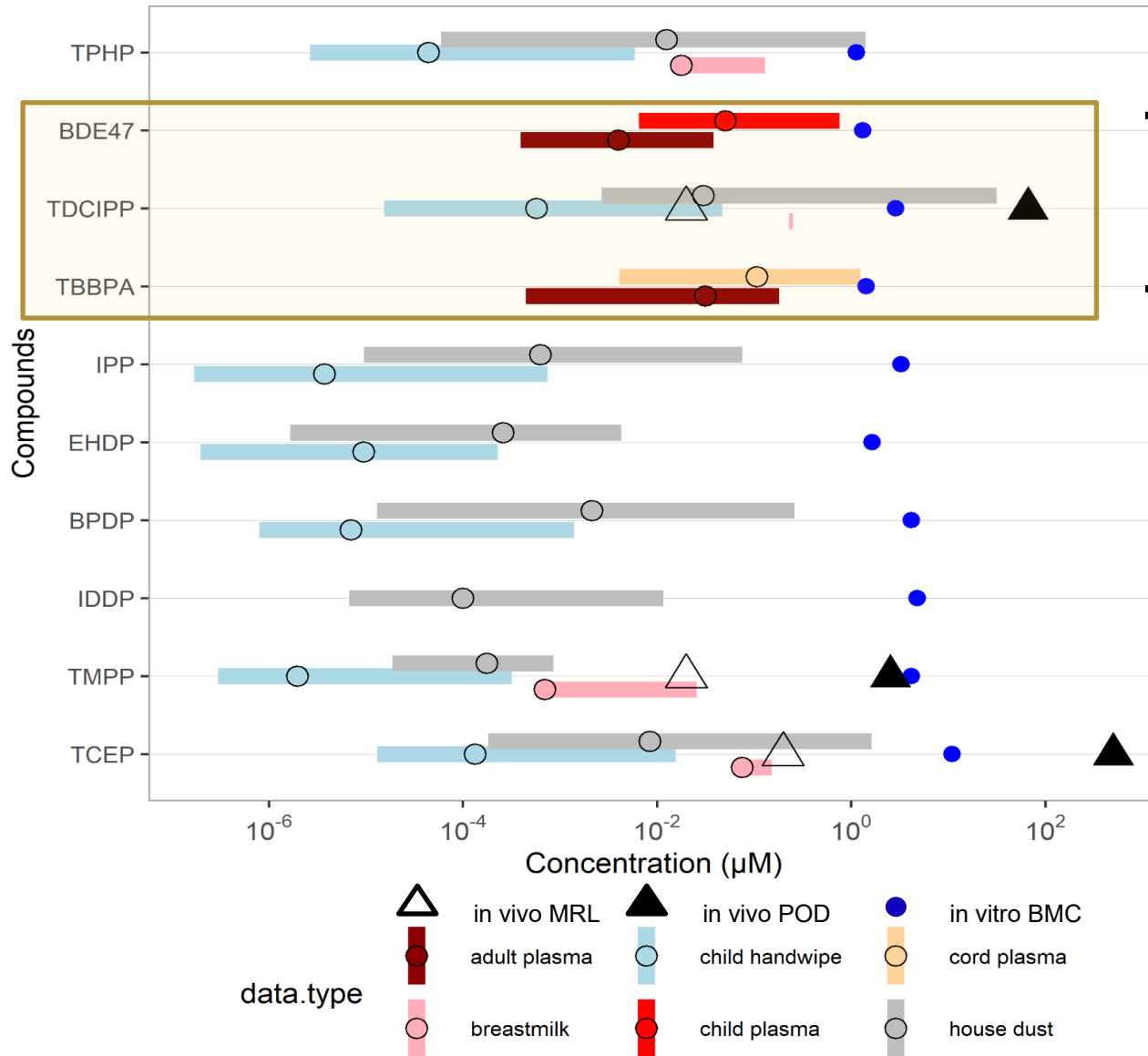
Relevance to Human Exposures



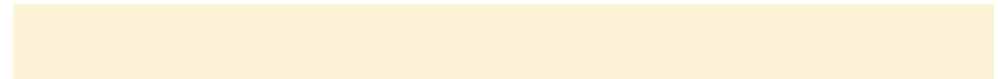
Updated figure from
Blum et al., 2019, Environ. Sci. Technol.



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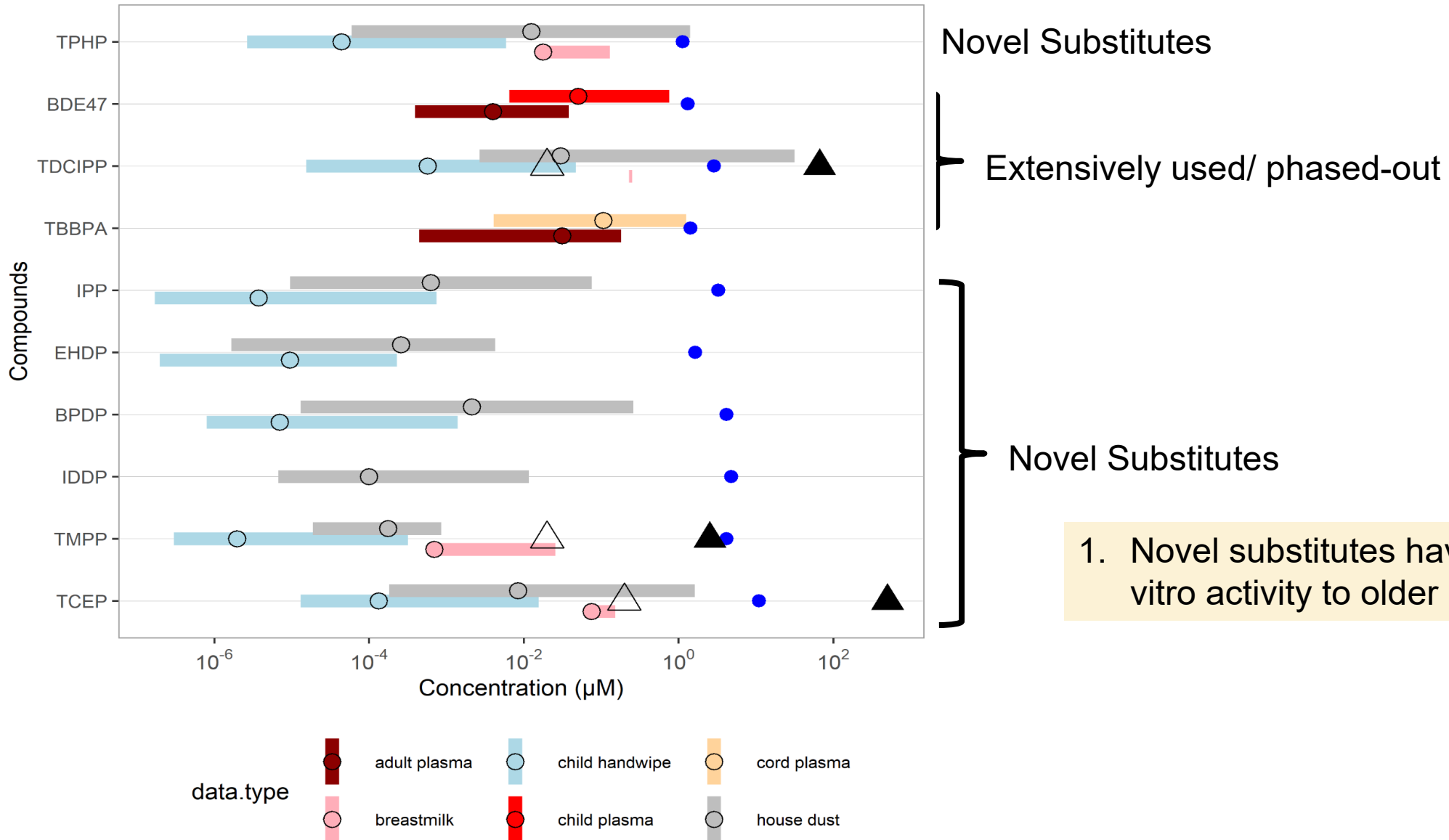


Extensively used/ phased-out





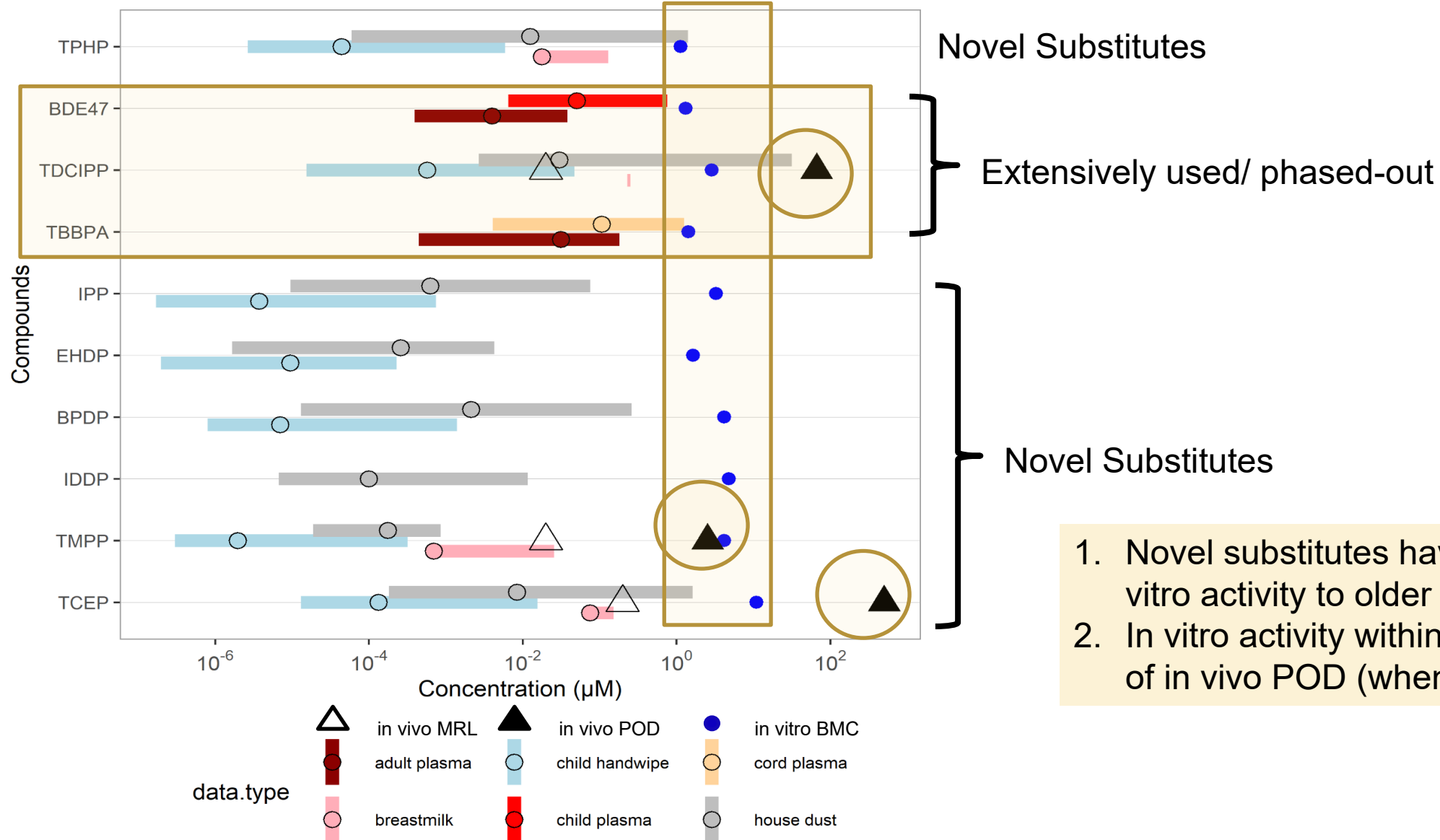
Relevance to Human Exposures



1. Novel substitutes have comparable in vitro activity to older FRs



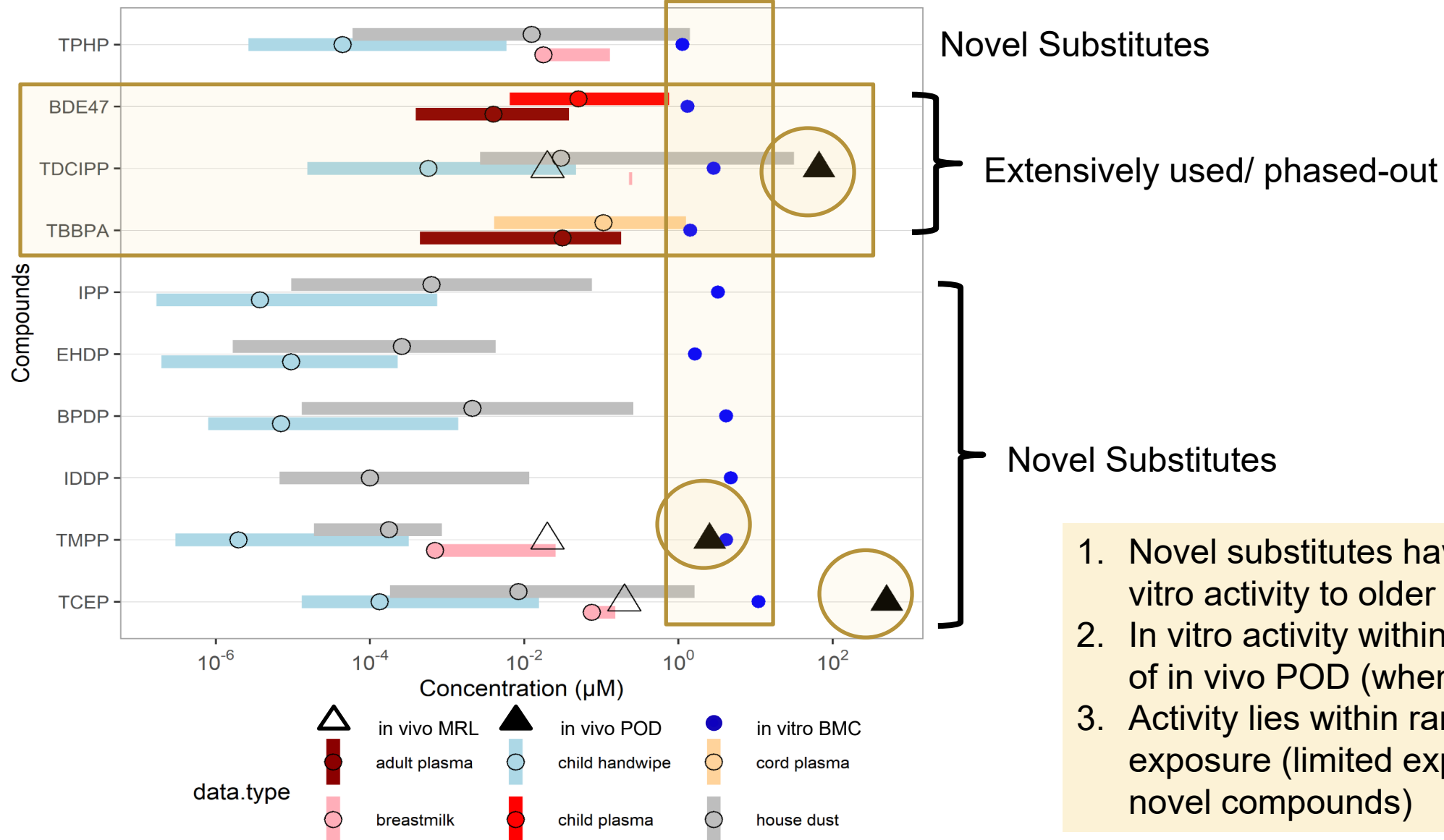
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2. In vitro activity within order of magnitude of in vivo POD (when known)



Relevance to Human Exposures



1. Novel substitutes have comparable in vitro activity to older FRs
2. In vitro activity within order of magnitude of in vivo POD (when known)
3. Activity lies within range of human exposure (limited exposure data for novel compounds)



Consideration for further development of AOP

GLUTAMATE

Cellular and Organ Effects	Organism Effects	Human Effects ¹
Monolayer in vitro cell culture <ul style="list-style-type: none"> Reduced response to glutamate¹ 	Rodent in vivo <ul style="list-style-type: none"> Impaired learning and memory^{2*} 	<ul style="list-style-type: none"> Adverse impacts on cognitive development including early language ability, and fine motor skills² Adverse behavioral development including withdrawal, attention problems, depression, hyperactivity, and aggression³ Decrease in IQ and working memory⁴ Social behavioral problems including less responsible behavior and more externalizing behaviors⁵
3D in vitro cell culture <ul style="list-style-type: none"> Alteration in expression of glutamate NMDA receptor⁶ NAA and L aspartic decrease⁶ Reduced levels of glutamate⁶ 		
Rodent in vivo <ul style="list-style-type: none"> Disruption of glutamate^{7*} Disruption of NAA, creatine and lactic acid^{7*} Increased levels of glutamate^{8*} Neuronal death^{7,8*} 		

GABA (GAMMA-AMINO BUTYRIC ACID)

Cellular and Organ Effects	Organism Effects	Human Effects ¹
Monolayer in vitro cell culture <ul style="list-style-type: none"> Inhibition of GABA R⁹ 	Zebrafish <ul style="list-style-type: none"> Hyperactivity¹² 	<ul style="list-style-type: none"> Adverse impacts on cognitive development including early language ability, and fine motor skills² Adverse behavioral development including withdrawal, attention problems, depression, hyperactivity, and aggression³ Decrease in IQ and working memory⁴ Social behavioral problems including less responsible behavior and more externalizing behaviors⁵
3D in vitro cell culture <ul style="list-style-type: none"> Decrease in genes involved in GABA production and signaling⁶ Decrease in GABA neurotransmitter⁶ 	Rodent in vivo <ul style="list-style-type: none"> Impaired learning and memory⁷ Increased ambulatory behavior⁷ 	
Zebrafish <ul style="list-style-type: none"> Altered levels of GABA neurotransmitter^{10,11} 		
Rodent in vivo <ul style="list-style-type: none"> GABA antagonist^{13*} Disruption of GABA neurotransmitter^{7*} 		

OTHER NEUROTRANSMITTERS

Cellular and Organ Effects	Organism Effects	Human Effects ¹
Monolayer in vitro cell culture <ul style="list-style-type: none"> 2D: Increase in differentiation of dopaminergic neurons¹⁴ 	Zebrafish <ul style="list-style-type: none"> Vulnerability to anxiety-like behavior potentially due to decrease in dopamine¹⁵ 	<ul style="list-style-type: none"> Adverse impacts on cognitive development including early language ability, and fine motor skills² Adverse behavioral development including withdrawal, attention problems, depression, hyperactivity, and aggression³ Decrease in IQ and working memory⁴ Social behavioral problems including less responsible behavior and more externalizing behaviors⁵
3D in vitro cell culture <ul style="list-style-type: none"> Decrease in dopamine neurotransmitter⁹ 	Rodent in vivo <ul style="list-style-type: none"> Increased ambulatory behavior^{13*} 	
Zebrafish <ul style="list-style-type: none"> Dopamine levels decrease¹⁰ Dopamine and dopamine signaling related genes decreased¹⁵ Decreased serotonin and histamine levels¹² 		
Rodent in vivo <ul style="list-style-type: none"> Dopamine signaling altered^{13*} Disruption in serotonin pathways^{16,17} Serotonin levels increased¹⁶ 		

Beyond Cholinesterase Inhibition: Developmental Neurotoxicity of Organophosphate Ester Flame Retardants and Plasticizers

Heather B. Patisaul,¹ Mamta Behl,^{2,3} Linda S. Birnbaum,^{2,3,4} Arlene Blum,^{5,6} Miriam L. Diamond,⁷ Seth Rojello Fernández,⁵ Helena T. Hogberg,⁸ Carol F. Kwiatkowski,^{5,9} Jamie D. Page,¹⁰ Anna Soehl,⁵ and Heather M. Stapleton⁴

Patisaul et al., 2021 EHE

INFLAMMATION, GLIA ACTIVATION AND OXIDATIVE STRESS

Cellular and Organ Effects	Organism Effects	Human Effects ¹
Monolayer in vitro cell culture <ul style="list-style-type: none"> Increased glia/neuro ratio¹ Inflammatory response² 	Zebrafish <ul style="list-style-type: none"> Altered locomotor behavior⁶ 	<ul style="list-style-type: none"> Adverse impacts on cognitive development, including early language ability, and fine motor skills⁹ Adverse behavioral development including withdrawal, attention problems, depression, hyperactivity, and aggression¹⁰ Decrease in IQ and working memory¹¹ Social behavioral problems including less responsible behavior, and more externalizing behaviors¹²
3D in vitro cell culture <ul style="list-style-type: none"> Gliosis/activated astrocytes² Increased cytokine release³ 		
Zebrafish <ul style="list-style-type: none"> Oxidative stress⁴ Increased GFAP levels⁶ Decreased histamine levels^{4,5} 		
Rodent in vivo <ul style="list-style-type: none"> Oxidative stress^{7,8} Microglia mediated inflammation² Increase in proinflammatory cytokines^{7*} 		

NEURONAL MORPHOLOGY AND FUNCTION

Cellular and Organ Effects	Organism Effects	Human Effects ¹
Monolayer in vitro cell culture <ul style="list-style-type: none"> Decrease in neurite out-growth^{13,14,15} Decreased neuronal network activity^{14,16} Cytotoxic to neural cells¹⁷ 	Zebrafish <ul style="list-style-type: none"> Altered locomotor behavior^{5,6,18,19} 	<ul style="list-style-type: none"> Adverse impacts on cognitive development, including early language ability, and fine motor skills⁹ Adverse behavioral development including withdrawal, attention problems, depression, hyperactivity, and aggression¹⁰ Decrease in IQ and working memory¹¹ Social behavioral problems including less responsible behavior, and more externalizing behaviors¹²
3D in vitro cell culture <ul style="list-style-type: none"> Decrease in expression of neurite skeleton genes³ 3D: Decreased expression of genes involved in synaptogenesis³ 		
Zebrafish <ul style="list-style-type: none"> Decrease in genes involved in cytoskeleton organization^{5,6} Synaptogenesis marker altered^{5,6} 		

Example of using KEs + underlying mechanistic data in the absence of MIEs

ENDOCRINE DISRUPTION

Cellular and Organ Effects	Organism Effects	Human Effects
Monolayer in vitro cell culture <ul style="list-style-type: none"> Antagonist and/or agonist for human hormone receptors¹² Increased estradiol and testosterone levels² Upregulation of genes involved in thyroid synthesis³ PPARY1 agonist⁴⁻⁸ 	Zebrafish <ul style="list-style-type: none"> Vulnerability to anxiety-like behavior in females¹⁴ 	<ul style="list-style-type: none"> Altered levels of TSH¹⁵ Thyroid hormone disruption¹⁶ Disruption of sex steroids and sex steroid binding globulins¹⁷
Zebrafish <ul style="list-style-type: none"> Thyroxine and T3 decreased in plasma⁷ Increase in T3 and T4³ Alteration of steroidogenesis, and estrogen metabolism² Alteration in genes involved in thyroid metabolism³ 	Rodent in vivo <ul style="list-style-type: none"> Sex differences in activity and anxiety behavior¹⁰⁻¹³ 	
Rodent in vivo <ul style="list-style-type: none"> Altered gene expression linked to endocrine disruption⁸ Increased serum thyroxine levels^{9,10} Endocrine disruption⁹ 		



- Overall, evidence available and the approach taken in this IATA case study allowed to achieve an acceptable level of certainty in prioritization of compounds for further testing
- It also allowed for DNT hazard identification and characterization of the OPFRs which was one of the purposes of the assessment.
- The analysis could likely be used by organizations like the CPSC to prioritize compounds for further testing and use the mechanistic data generated here as weight of evidence.



- Reduce uncertainty levels
 - Anchor data to AOPs
 - Confirm IVIVE models with in vivo data
 - Harmonization of protocols
 - Transferability of assays
 - Understand bioavailability in the different assays
- Explore ways of weighting the different assays
 - How many assays need to be positive for different regulatory purposes?
 - Should assays with higher biological activity be given more weight?
- Develop assays for key events currently missing
 - E.g., Myelination, differentiation and proliferation of astrocytes and microglia, ontogeny of neurotransmitters and receptors

Human iPSC-Derived Neural Model

Many of the major cell types of the CNS

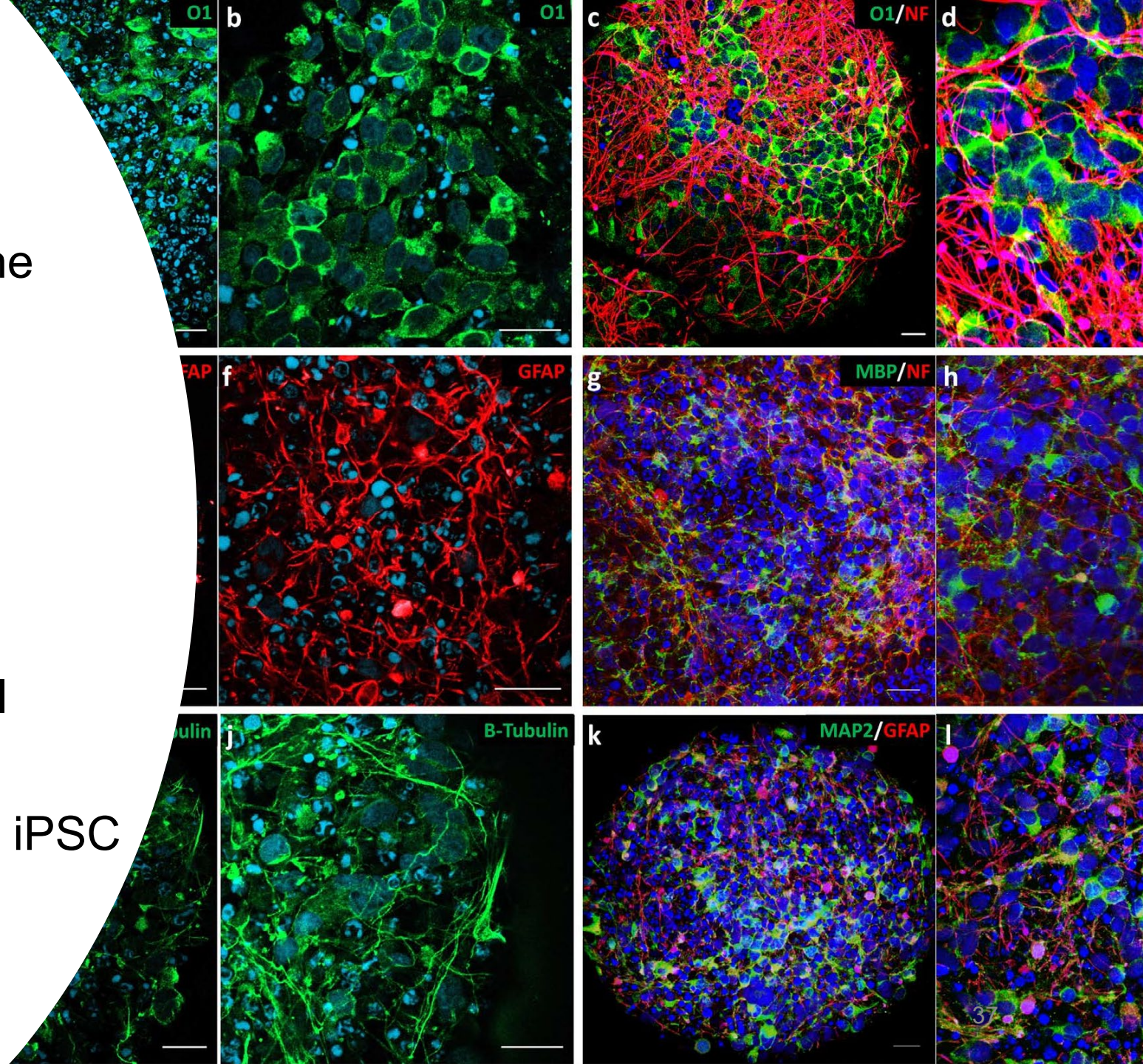
Microglia can be added

350-500 μm diameter

Reproducible in size and cell composition

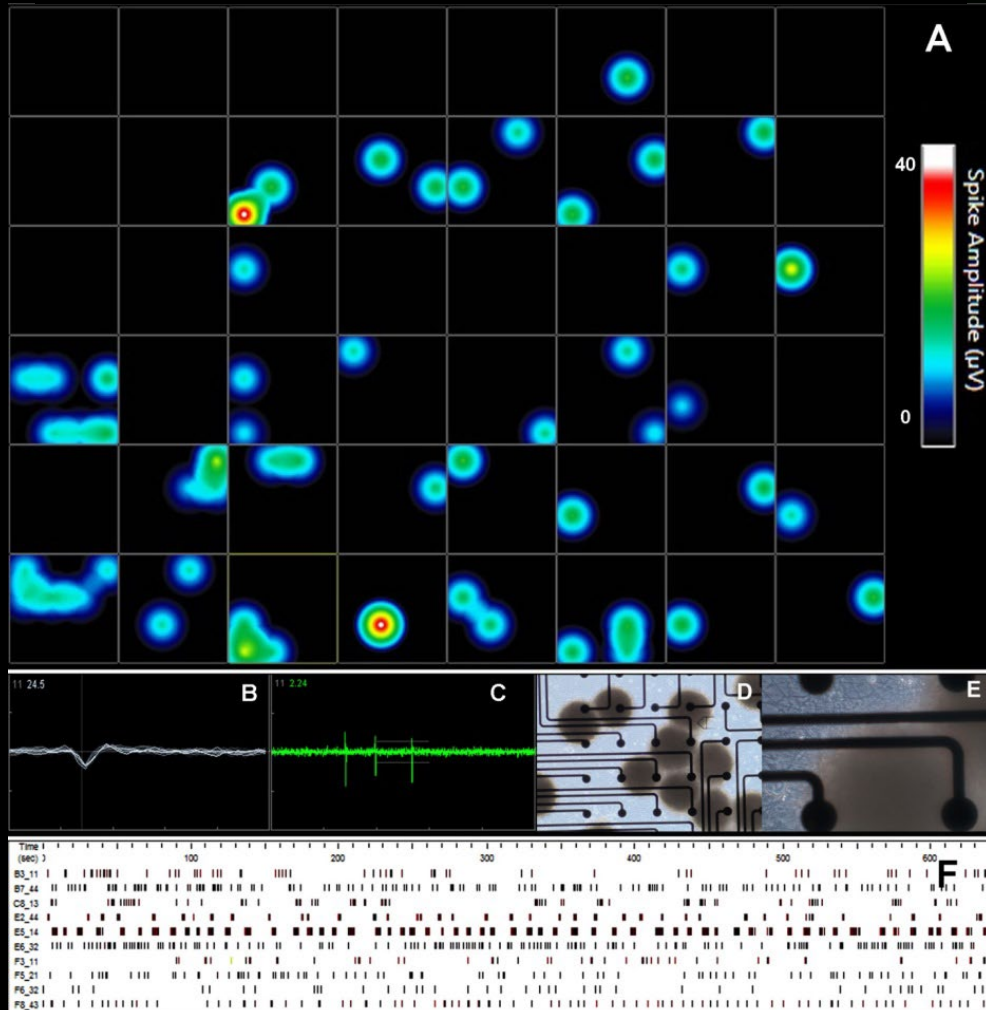
Shown critical elements of neural development

Genetic background from patient iPSC

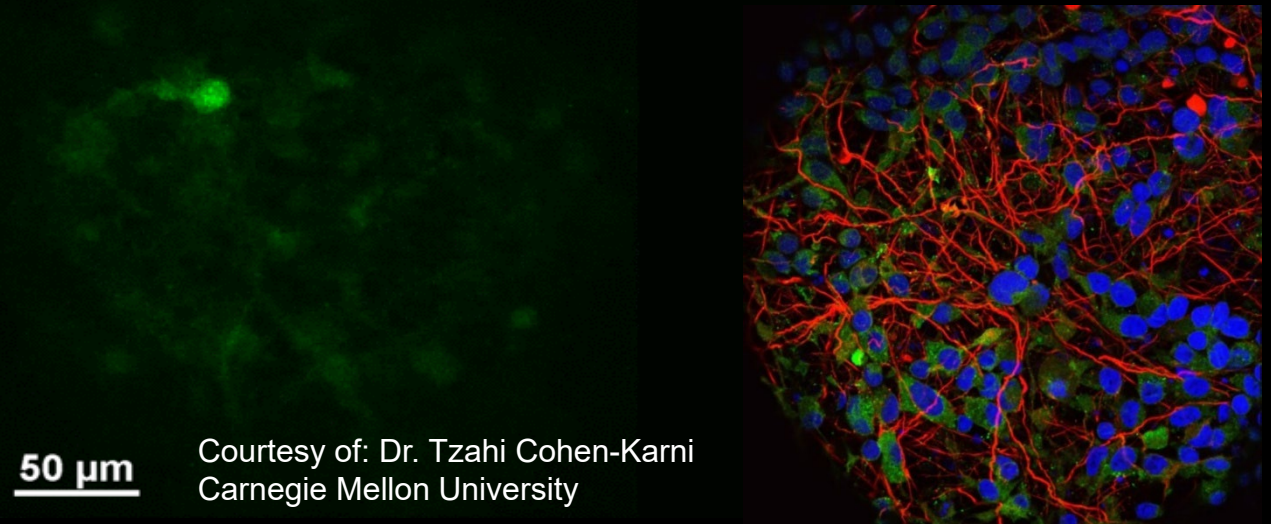


Functional neurons and glial cells

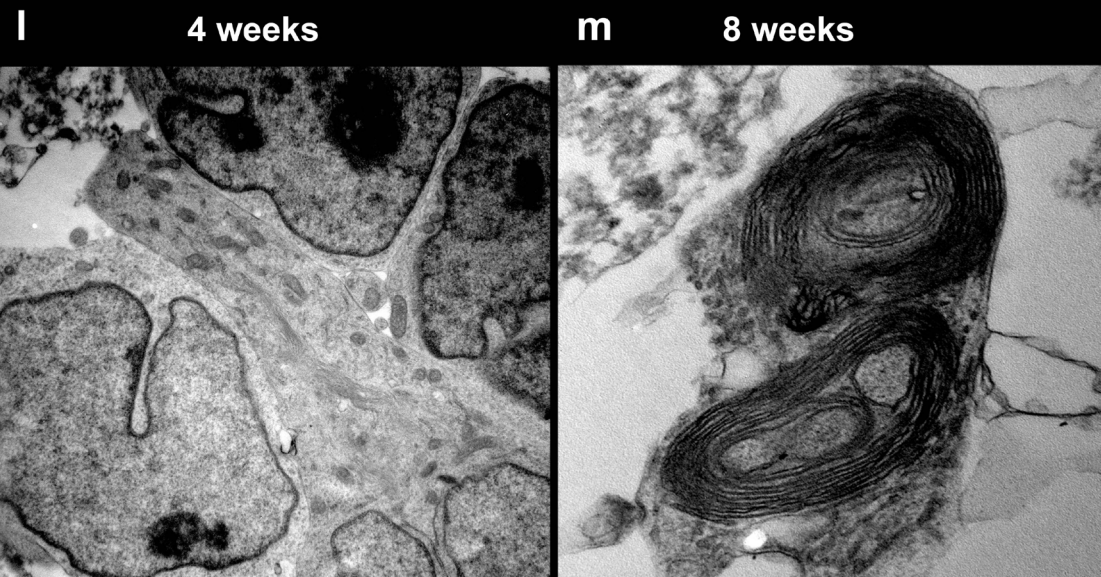
Micro-electrode arrays to measure spontaneous electrical activity



Ca^{2+} signaling



Electron microscopy





Stakeholders nominated chemical library

Selection Criteria

- Evidence of DNT *in vivo*
- Known human exposure
- Guideline study complete, lacking *in vitro*
- Suggested by multiple stakeholders
- Incomplete *in vitro* battery data

Phase 1: 115 chemical set
Currently tested in the battery in the individual labs



Assay development in vitro and in vivo

> [Environ Health Perspect.](#) 2021 Apr;129(4):47015. doi: 10.1289/EHP8314. Epub 2021 Apr 30.

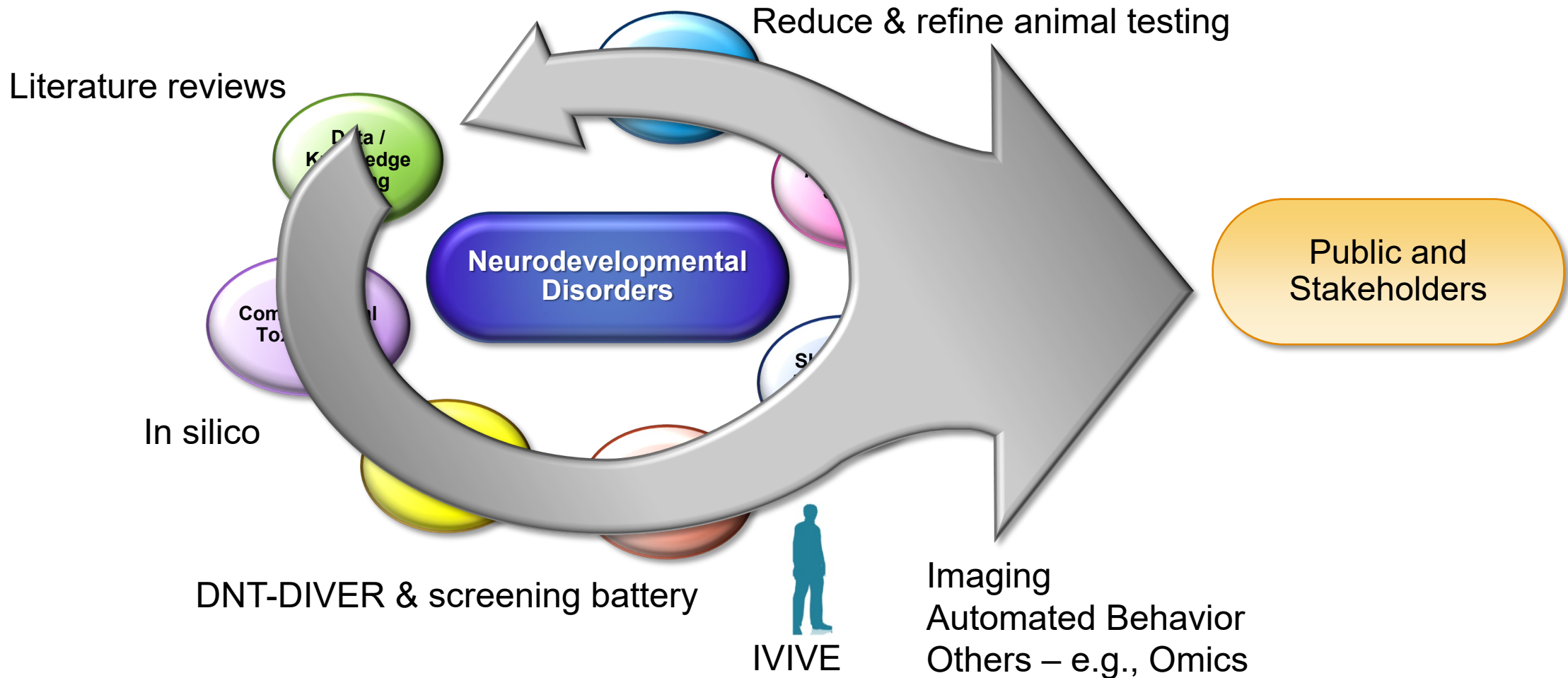
Assessing the Association of Mitochondrial Function and Inflammasome Activation in Murine Macrophages Exposed to Select Mitotoxic Tri-Organotin Compounds

Gabrielle M Childers ¹, Caroline A Perry ¹, Barbara Blachut ¹, Negin Martin ², Carl D Bortner ³, Stella Sieber ⁴, Jian-Liang Li ⁵, Michael B Fessler ⁶, G Jean Harry ¹

Linking mechanistic bioactivity
to clinical end-points



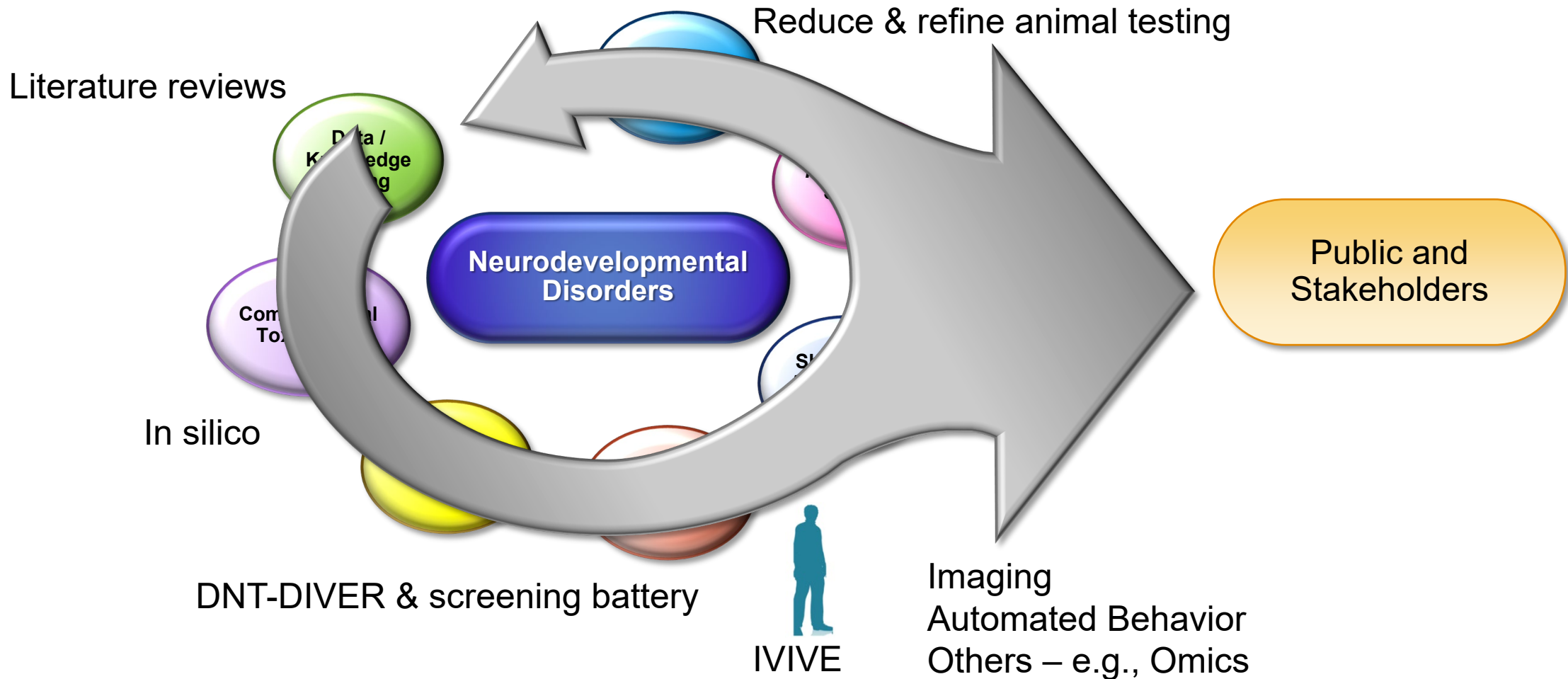
DNT HEI in DNTP's Translational Toxicology Pipeline





DNT HEI in DNTP's Translational Toxicology Pipeline

Ultimate goal is to more effectively predict DNT for unknown environmental chemicals to prevent neurodevelopmental disorders





The NICEATM Group

Speaker View Exit Full Screen

Judy Strickland Nicole Kleinstreuer Jaleh Abedini Dave Allen John Rooney

Pei-Li Yao Amber Daniel Bethany Cook Xiaoqing Chang Agnes Karmaus

Patricia Ceger Alex Borrel Jon Hamm Cathy Sprankle Lauren Browning

Arpit Tandon Eric McAfee Jason Phillips Shannon Bell Steven Morefield

David Hines Matt Stout Kamel Mansouri Ruchir Shah Neepa Choksi helena

Mute Stop Video Security Participants 25 Chat Share Screen Polling Record Reactions End