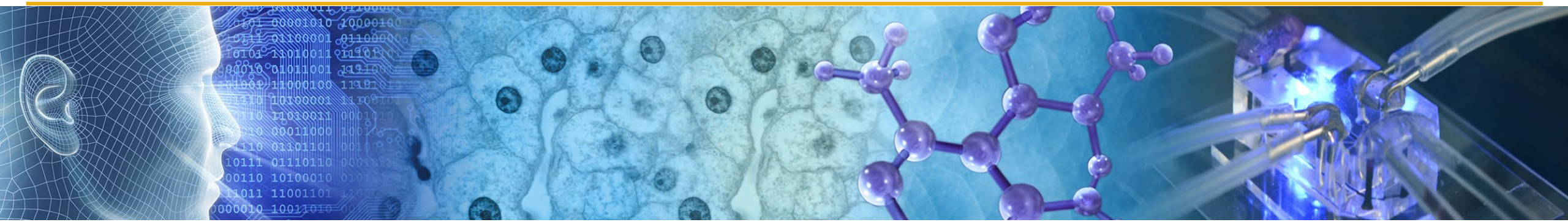
NTP
National Toxicology Program



Developmental Neurotoxicity Assessment Using In Vitro Assays

Helena Hogberg
Division of the NTP, National Institute of Environmental Health Sciences
NTP Interagency Center for the Evaluation of
Alternative Toxicological Methods (NICEATM)

UNITED STATES ICCVAM Advancing Alternatives to Animal Testing

DNT HEI Program Management Team

DNTP

Mamta Behl*
Former Team Lead

Jeremy Erickson
ODS

Laura Hall
OPO

Chris McPherson
MTB

Robert Sills
CMPB, Liaison

Shannah Witchey
STB

Neurobiology Core
DIR

Jesse Cushman
NL/DIR

Briana Bernstein
NL/DIR (adjunct)

Leslie Wilson
NL/DIR (adjunct)

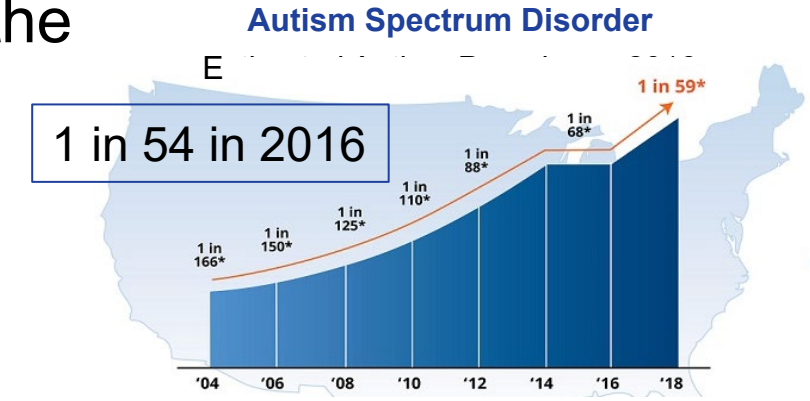
Division of the National Toxicology Program (DNTP)
ODS (Office of Data Science)
OPO (Office of Program Operations)
CMPB (Comparative & Molecular Pathogenesis Branch)
MTB (Mechanistic Toxicology Branch)
STB (Systems Toxicology Branch)

Division of Intramural Research (DIR)
NL (Neurobiology Laboratory)

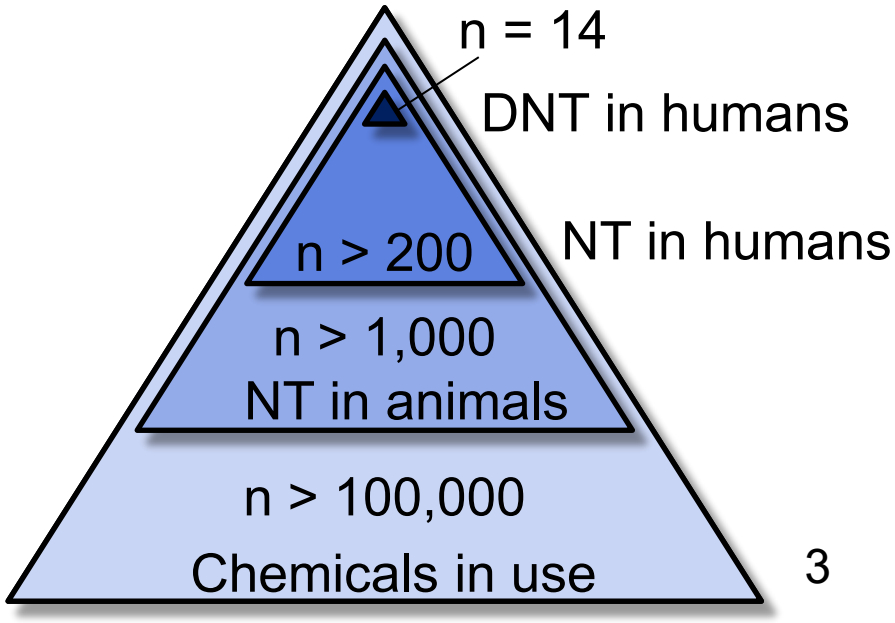
*Now Neurocrine Biosciences

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"



Revised from Grandjean and Landrigan 2006, Lancet

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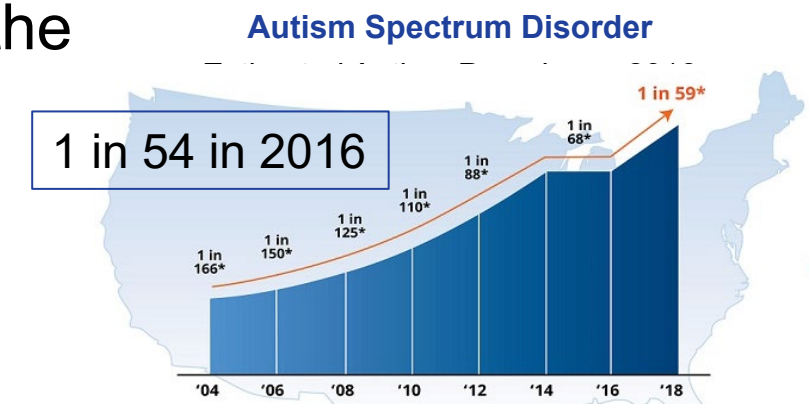

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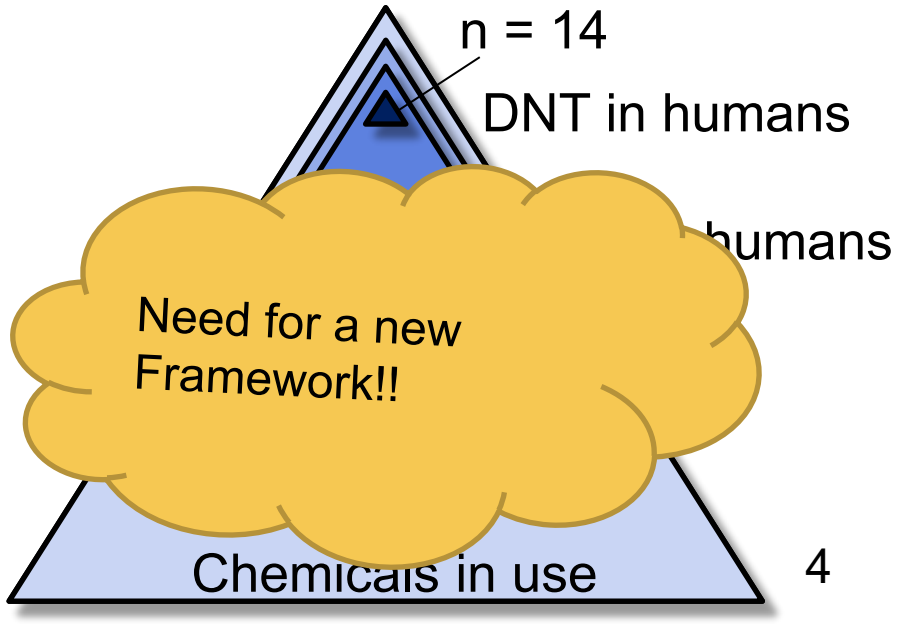

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Regulatory Focus on Developing New Frameworks

Perspectives | Brief Communication

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

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

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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 Bennekou[i], Pamela J. Lein[j], Marcel Leist[k], William R. Mundy[l], Martin Paparella[m], Aldert H. Piersma[n], 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,*]

OXFORD

SOT | Society of Toxicology

www.toxsci.oxfordjournals.org

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

doi: 10.1093/toxsci/kfy211

Advance Access Publication Date: November 23, 2018

Forum

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‖

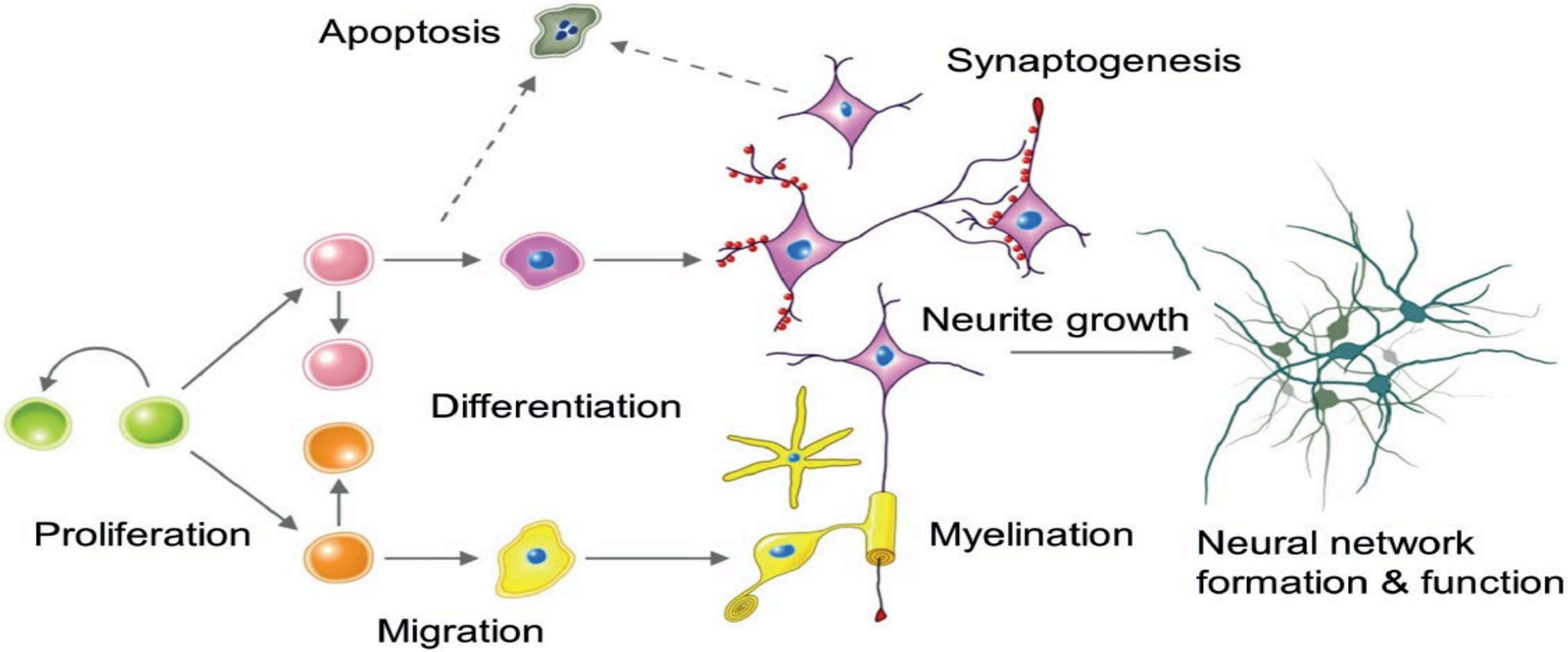
FIFRA Scientific Advisory Panel Meeting Minutes and Final Report No. 2020-02

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

In vitro Assays to Model Neurodevelopmental Key Events



Aschner et al., 2017, ALTEX

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

Bal-Price et al., 2018, ALTEX

f4 Workshop Report*

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

Anna Bal-Price [1], Helena T. Hogberg [2], Kevin M. Crofton [3], Mardas Daneshian [4], Rex E. FitzGerald [5], Ellen Fritsche [6], Tuula Heinonen [7], Susanne Hougaard Bennekou [8], Stefanie Klima [9], Aldert H. Piersma [10], Magdalini Sachana [11], Timothy J. Shafer [3], Andrea Terron [12], Florianne Monnet-Tschudi [5,13], Barbara Viviani [14], Tanja Waldmann [9], Remco H. S. Westerink [15], Martin F. Wilks [5], Hilda Witters [16], 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 (Neuri Tox)	3Dh	3Dr	2Dm	UKN5 (PeriTox)	ZFE

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NPC6	A	B	B	B+
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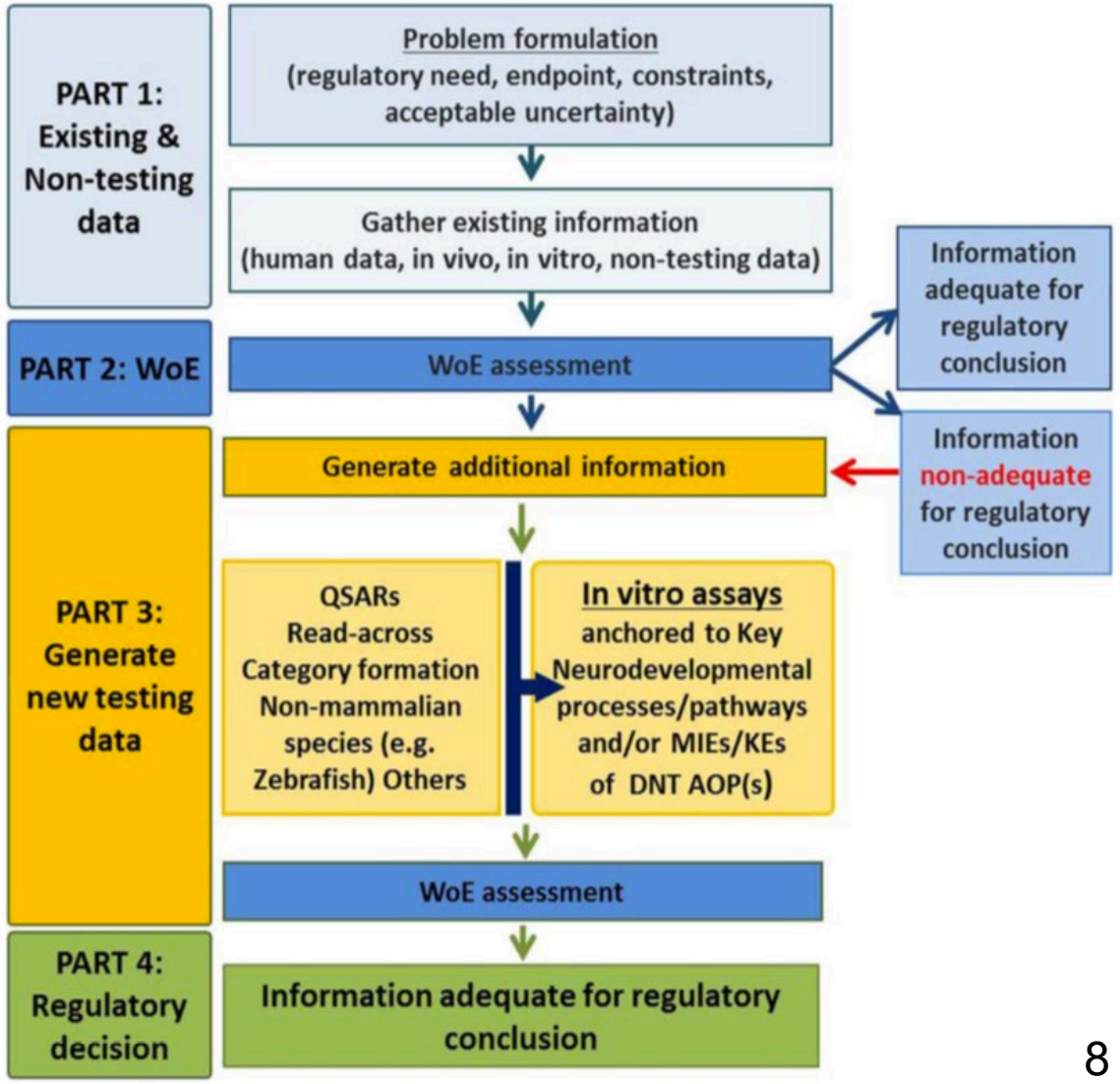
3Dr	A	A	A	A
3Dh	B	C	C	C+
ZFE	B	B	A	B+

Integrated Approach to Testing and Assessment (IATA) for DNT



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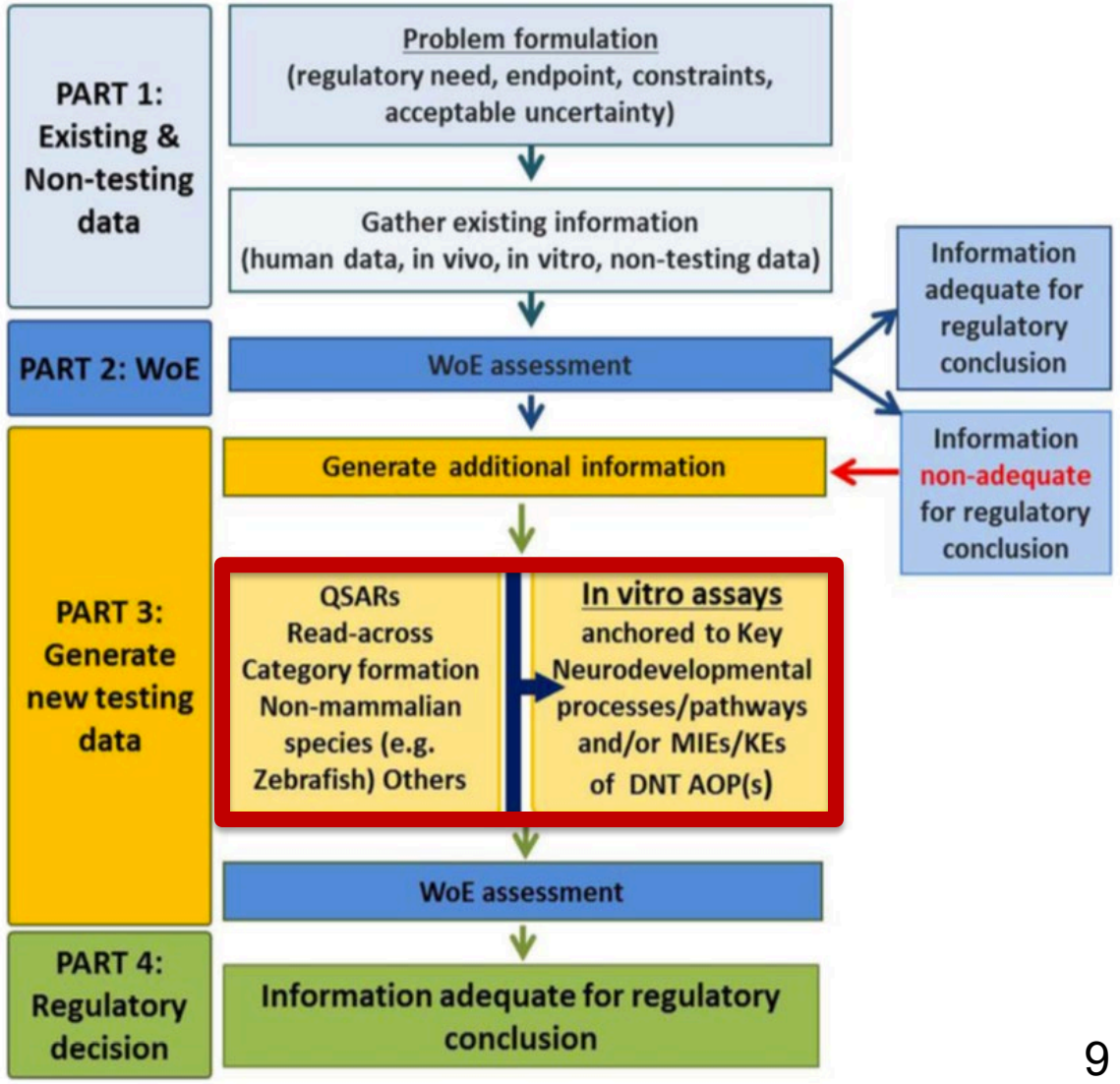
Integrated Approach to Testing and Assessment (IATA) for DNT



Regulatory Support and Application

Expert Group on DNT

Guidance Document

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

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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.

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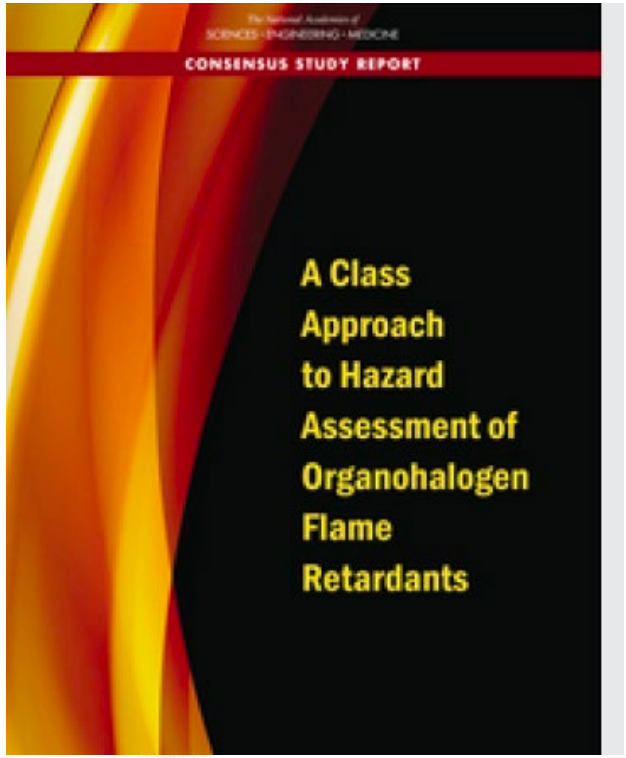
Need for class evaluation of Flame Retardants

- 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

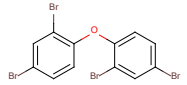
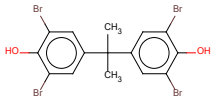
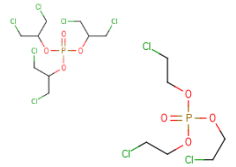
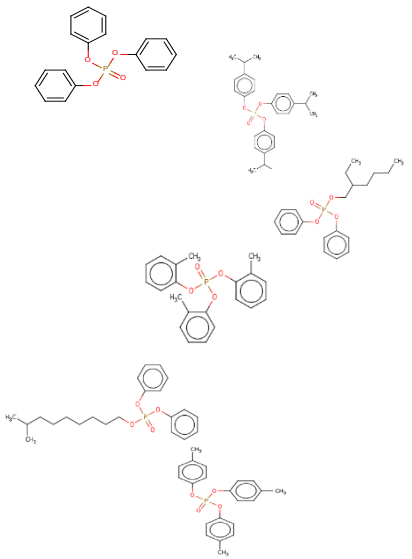


Class Evaluation of Flame Retardants (NAS 2019)

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

Chemicals in the IATA

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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29761-21-5	Isodecyl diphenyl phosphate	IDDP*	
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Phased-out (BDE)

Extensively used (and studied)

**representative isomer in mixture is shown as structure*

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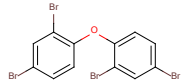
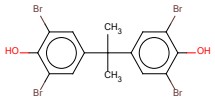
5436-43-1	2,2'4,4'-Tetrabromodiphenyl ether	BDE-47	
79-94-7	3,3',5,5'-Tetrabromobisphenol A	TBBPA	
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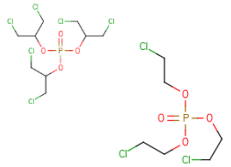
13674-87-8	Tris(1,3-dichloro-2-propyl)phosphate	TDCIPP	
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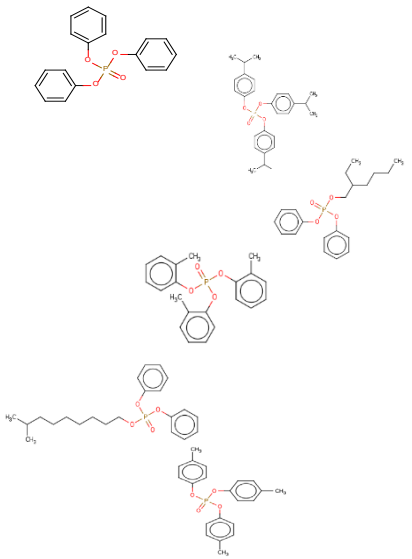
115-86-6	Triphenyl phosphate	TPHP	
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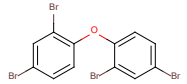
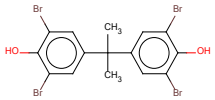
5436-43-1	2,2'4,4'-Tetrabromodiphenyl ether	BDE-47	
79-94-7	3,3',5,5'-Tetrabromobisphenol A	TBBPA	
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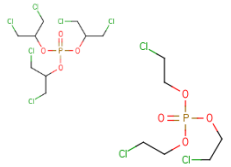
13674-87-8	Tris(1,3-dichloro-2-propyl)phosphate	TDCIPP	
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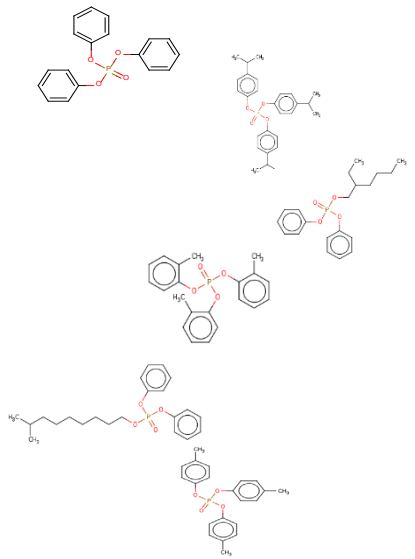
115-86-6	Triphenyl phosphate	TPHP	
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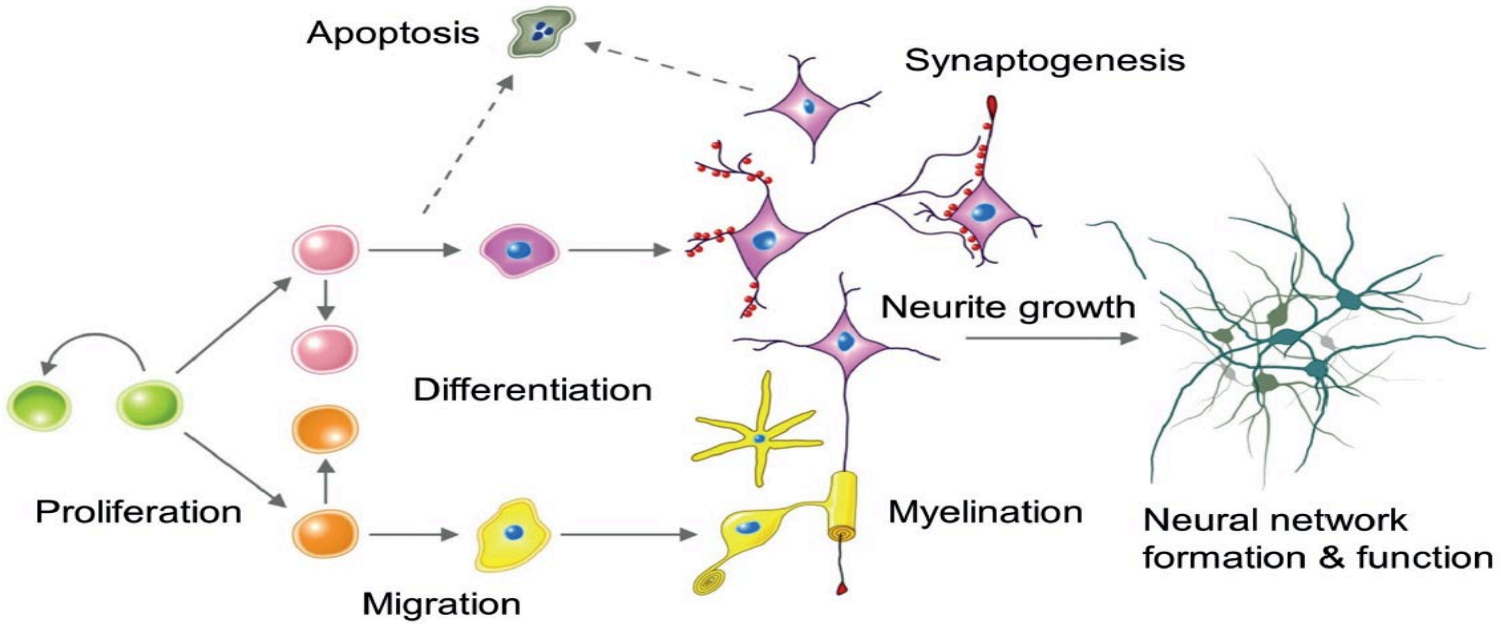
Aliphatic organohalogens

Aromatic phosphates (non-halogenated)

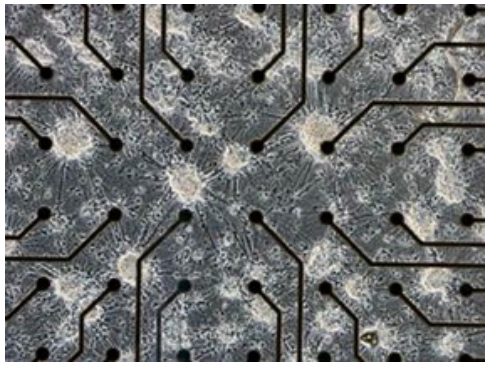
**representative isomer in mixture is shown as structure*

Battery of Tests Covers Key Neurodevelopmental Events

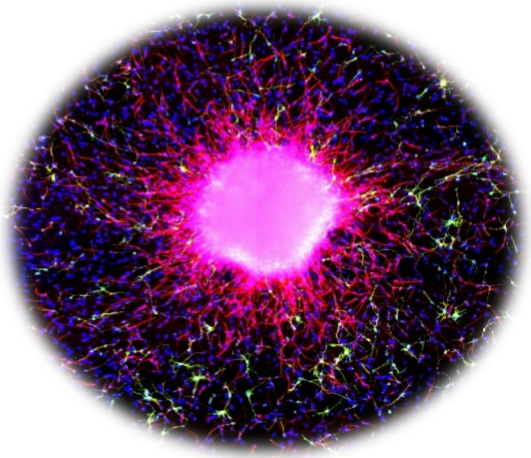


Cell lines, primary cells, stem cells, mainly human derived

2-D assays



3D- Neurospheres



Zebrafish

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

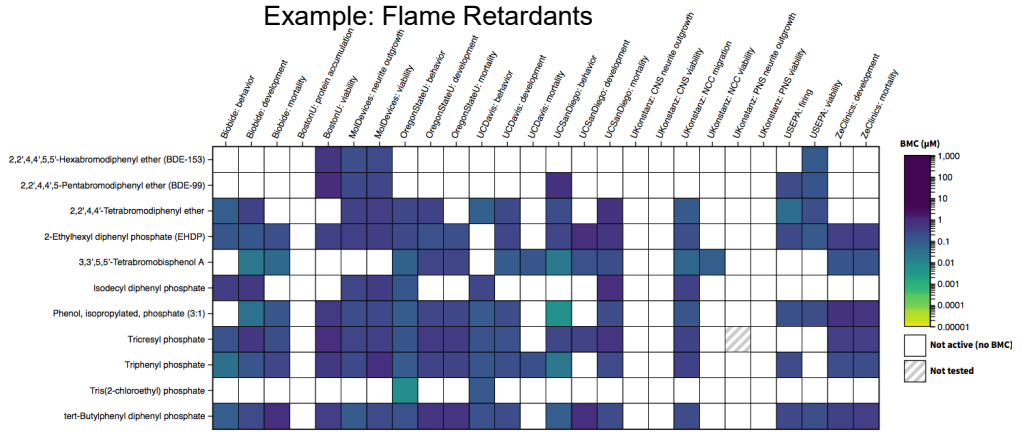
See DNT-DIVER https://sandbox.ntp.niehs.nih.gov/neurotox/

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?

Example: Flame Retardants

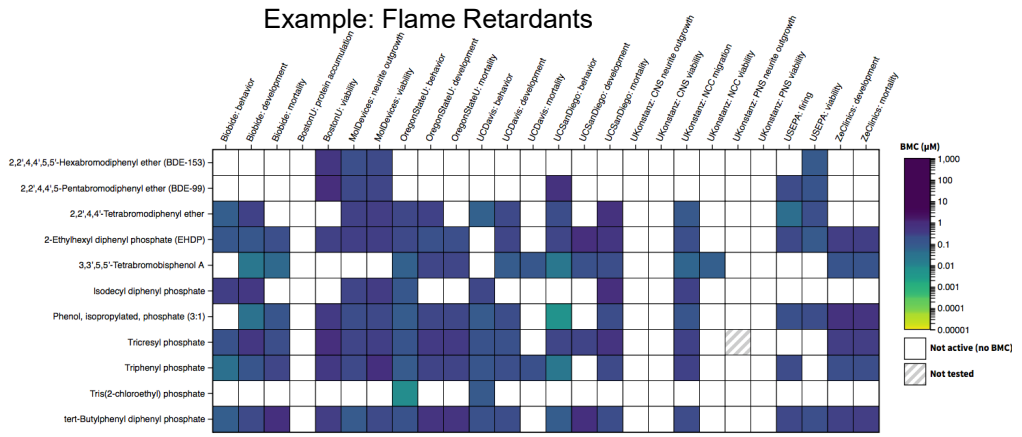


Compare activity of compounds/classes across multiple assays

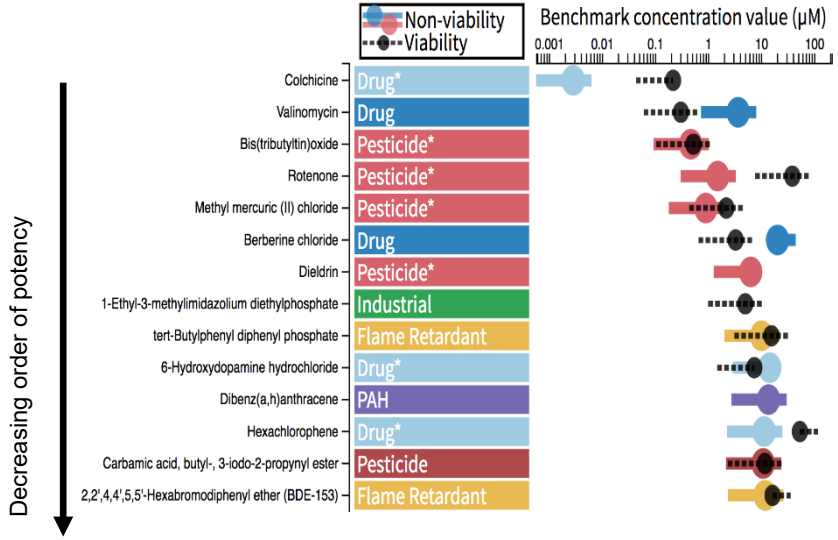
https://sandbox.ntp.niehs.nih.gov/neurotox/

DNT- Data Integration and Visualization Enabling Resource

What can you do in DNT-DIVER?



Compare activity of compounds/classes across multiple assays



Compare activity of compounds within an assay

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

DNT- Data Integration and Visualization Enabling Resource

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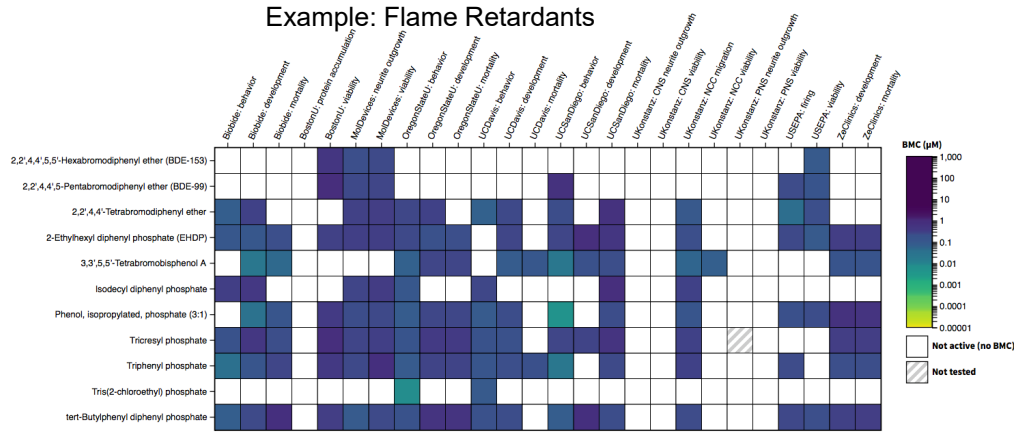
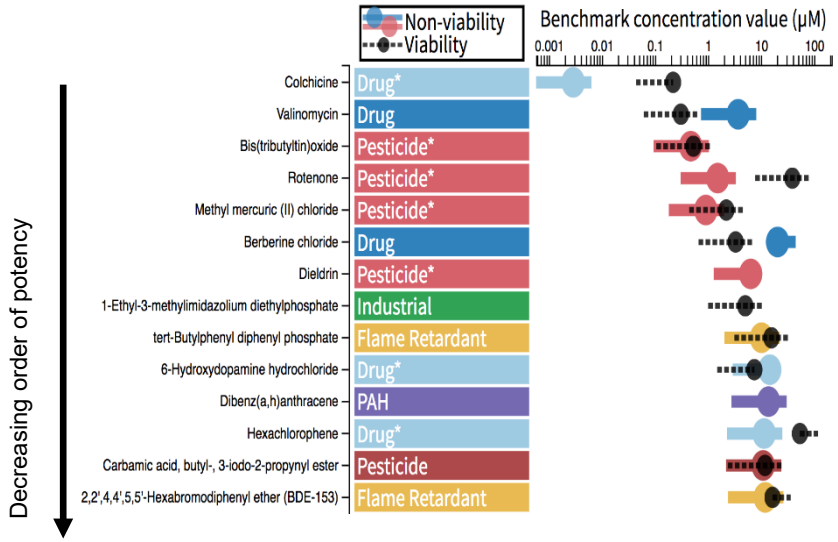
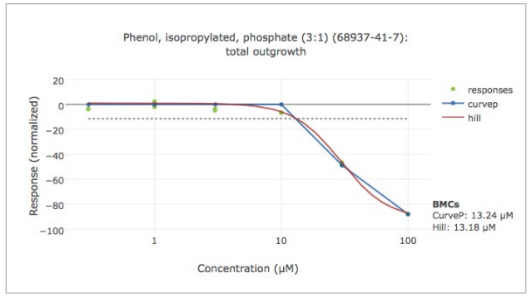


Compare activity of compounds/classes across multiple assays



Compare activity of compounds within an assay



Individual dose-response curves

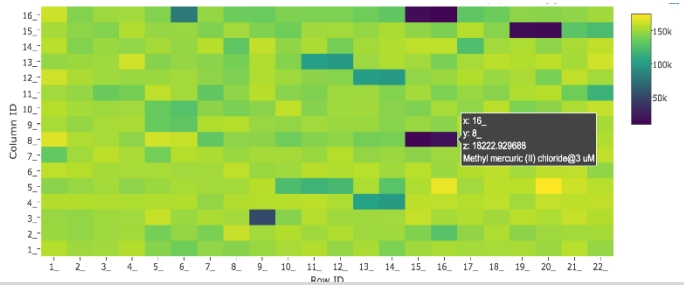
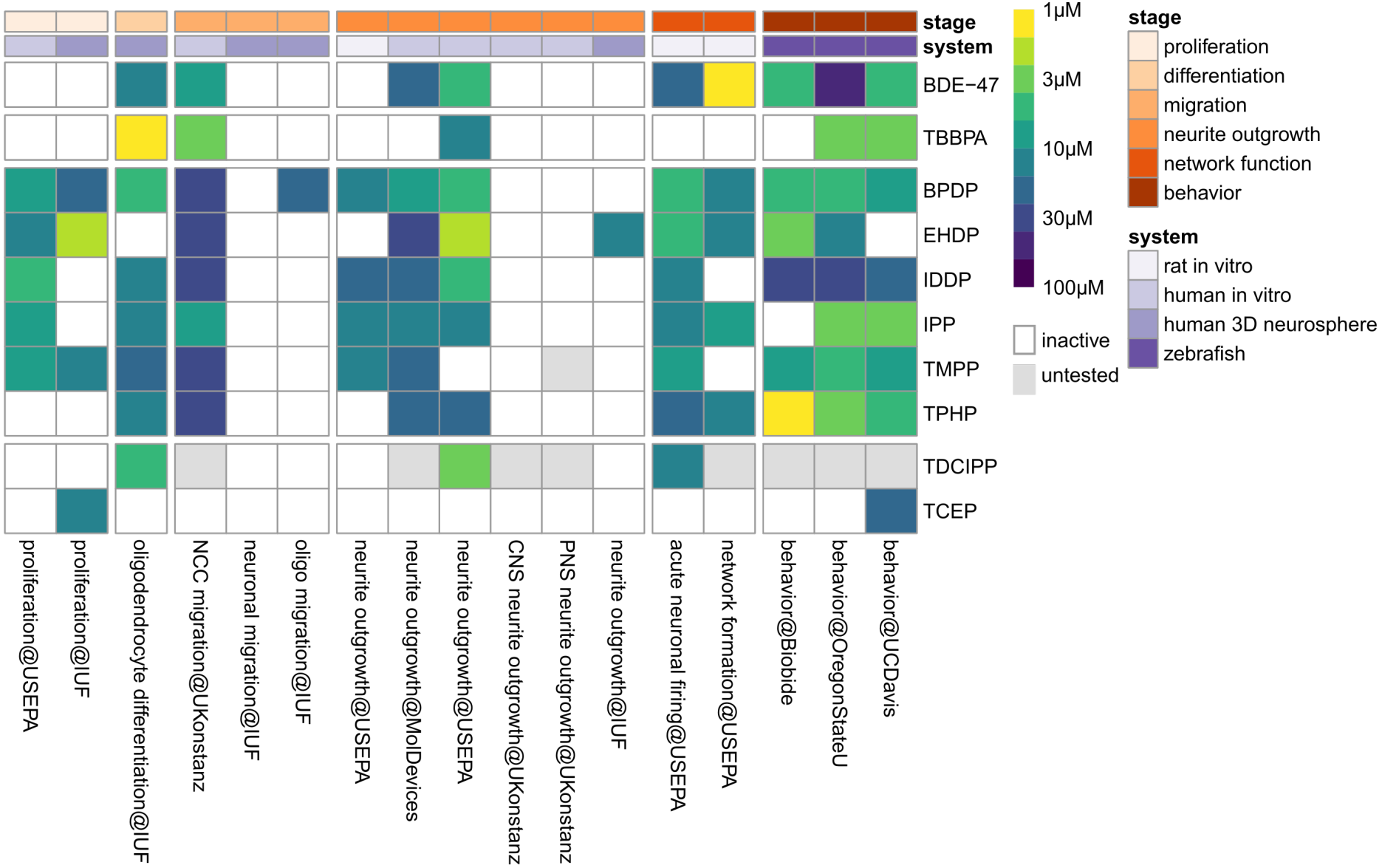


Plate and well level information

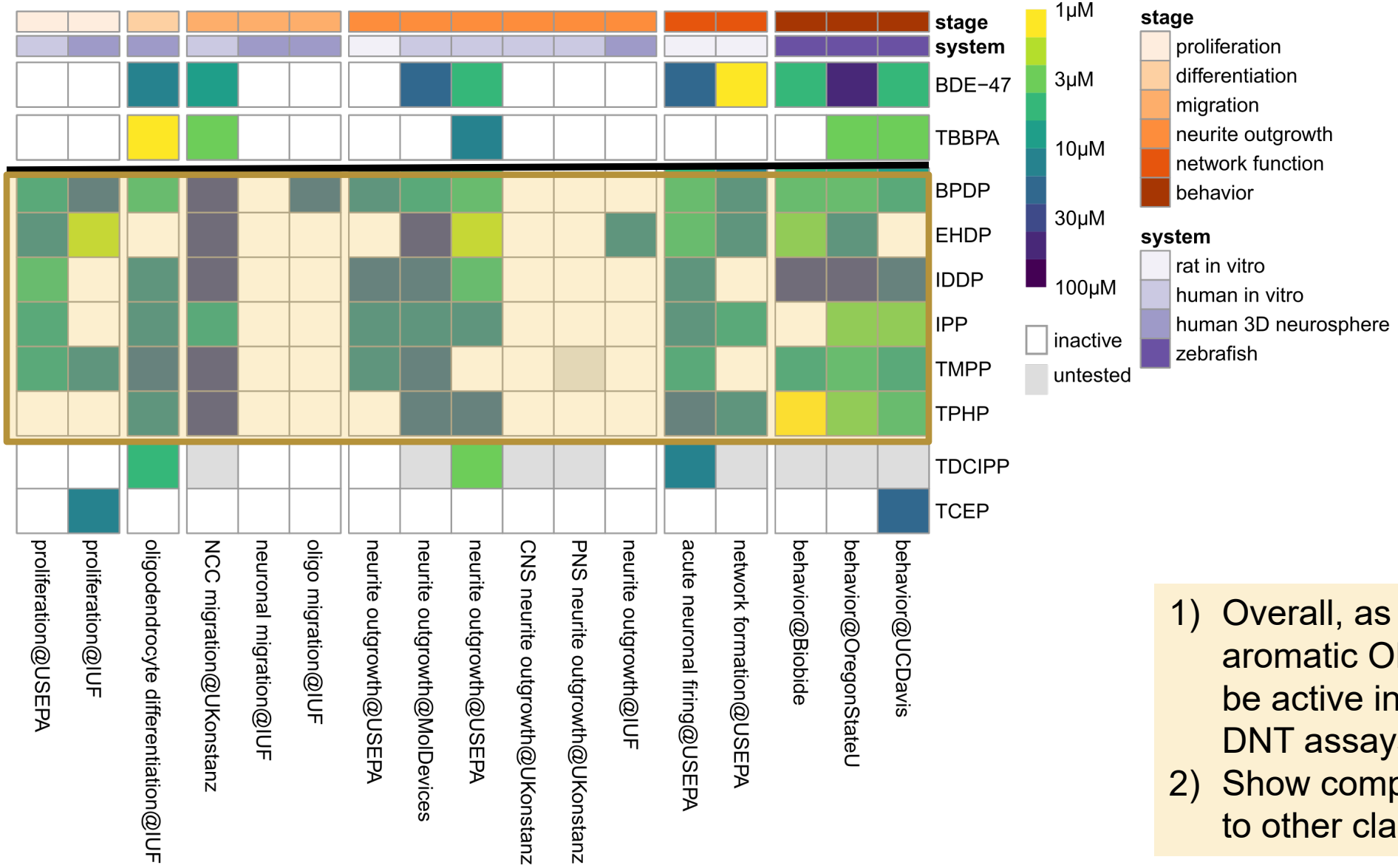
Control variability in assay

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

Summary of Findings

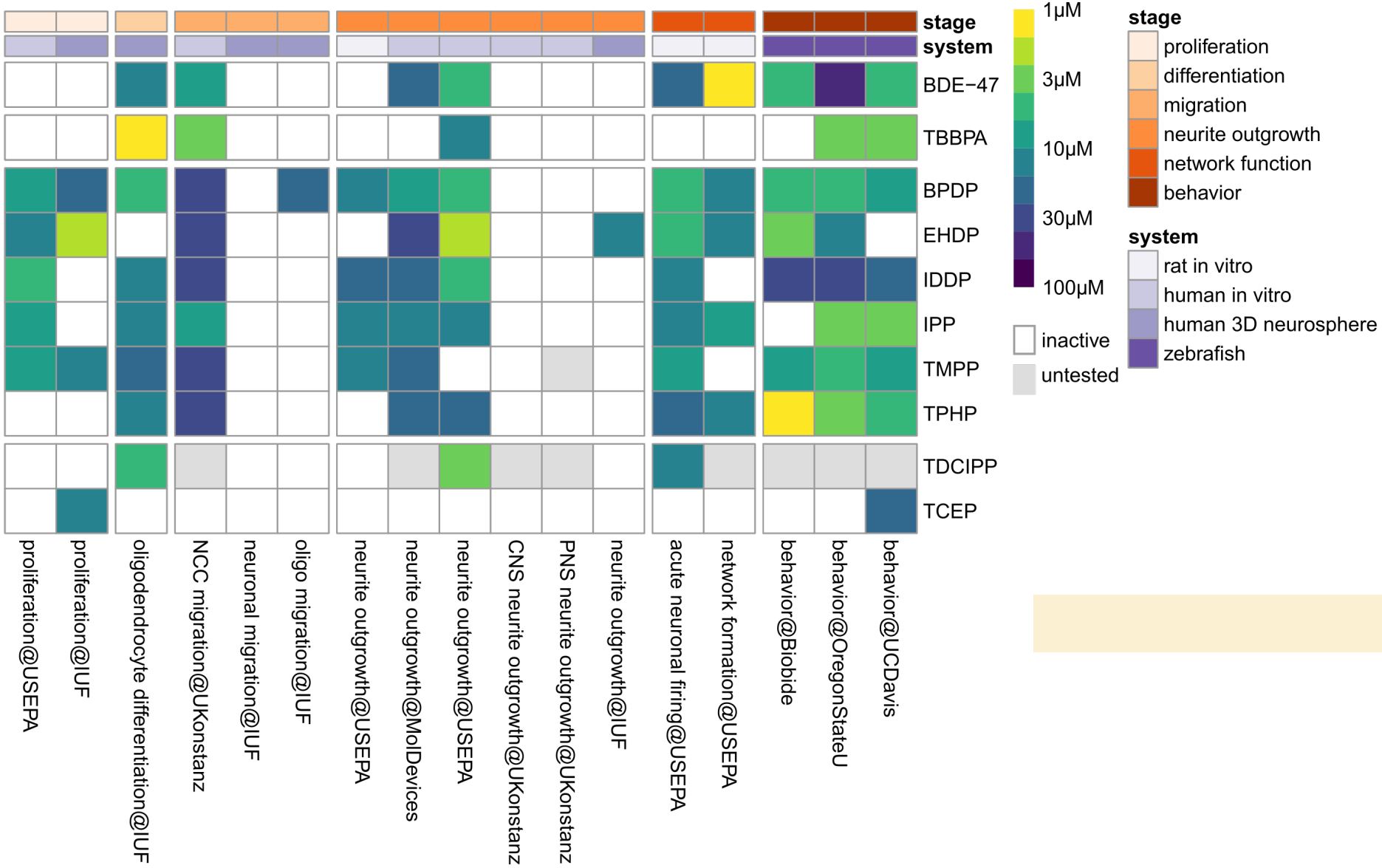


Summary of Findings



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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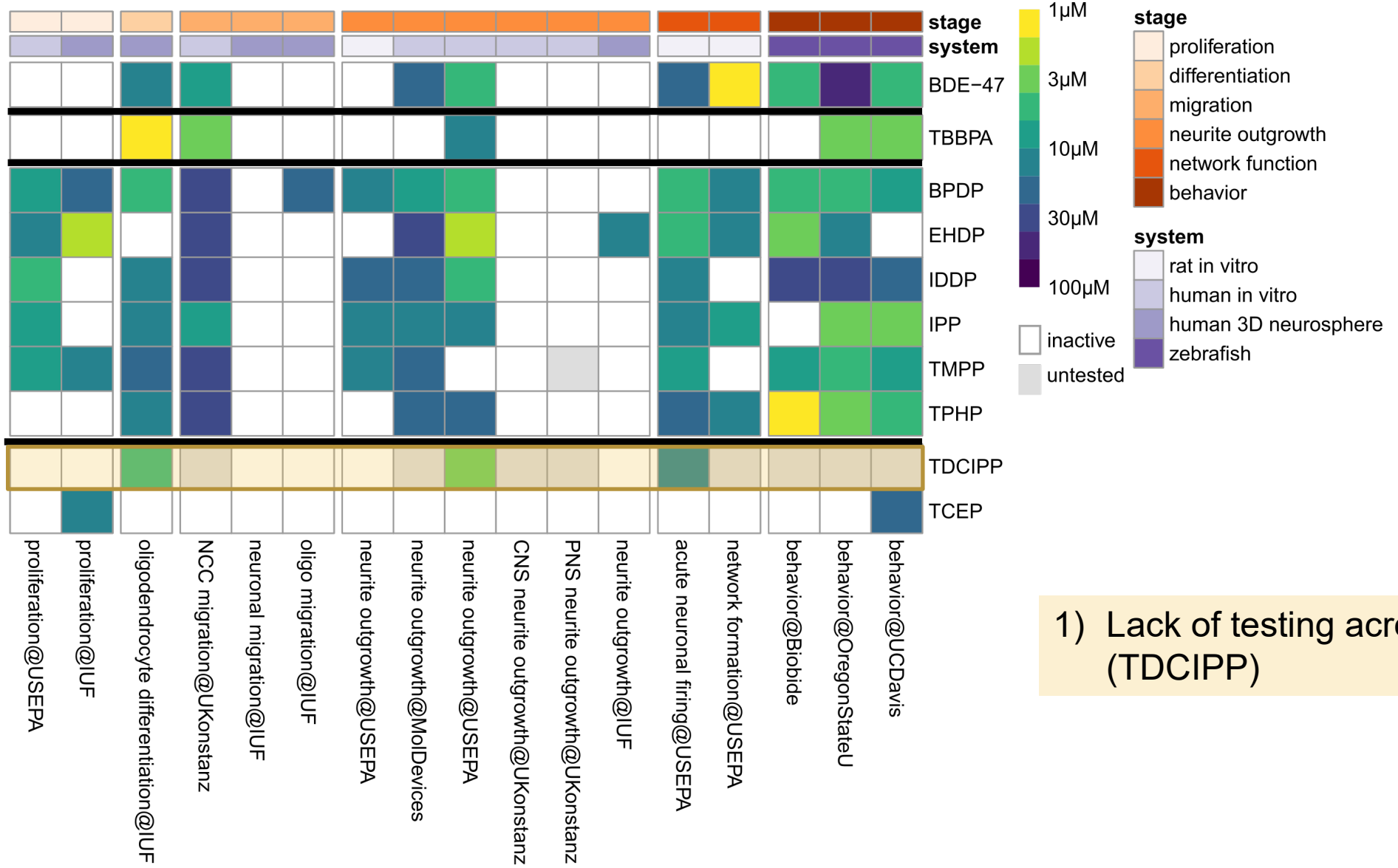
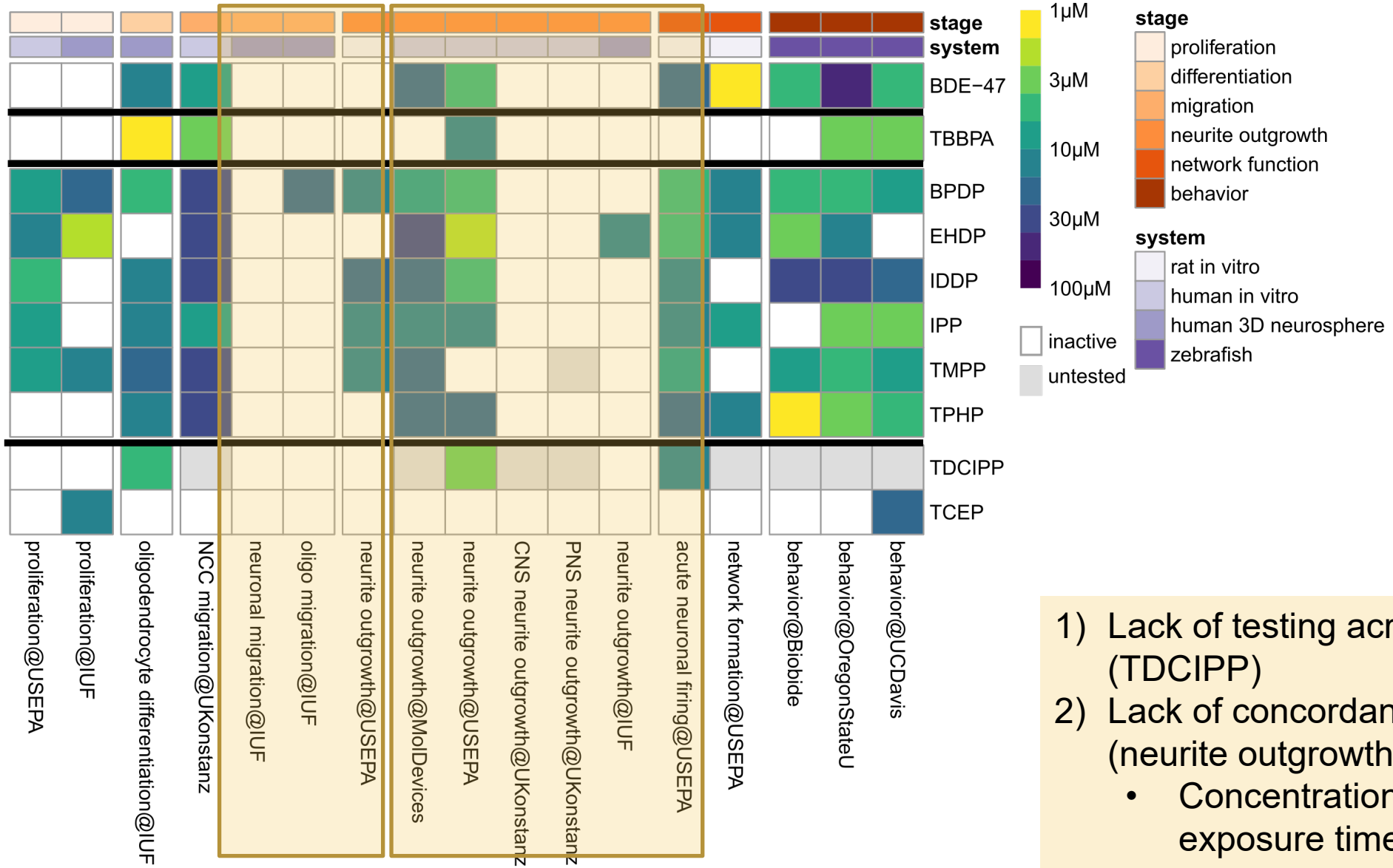


1) Lack of testing across assays (TDCIPP)

Sources of Uncertainty



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

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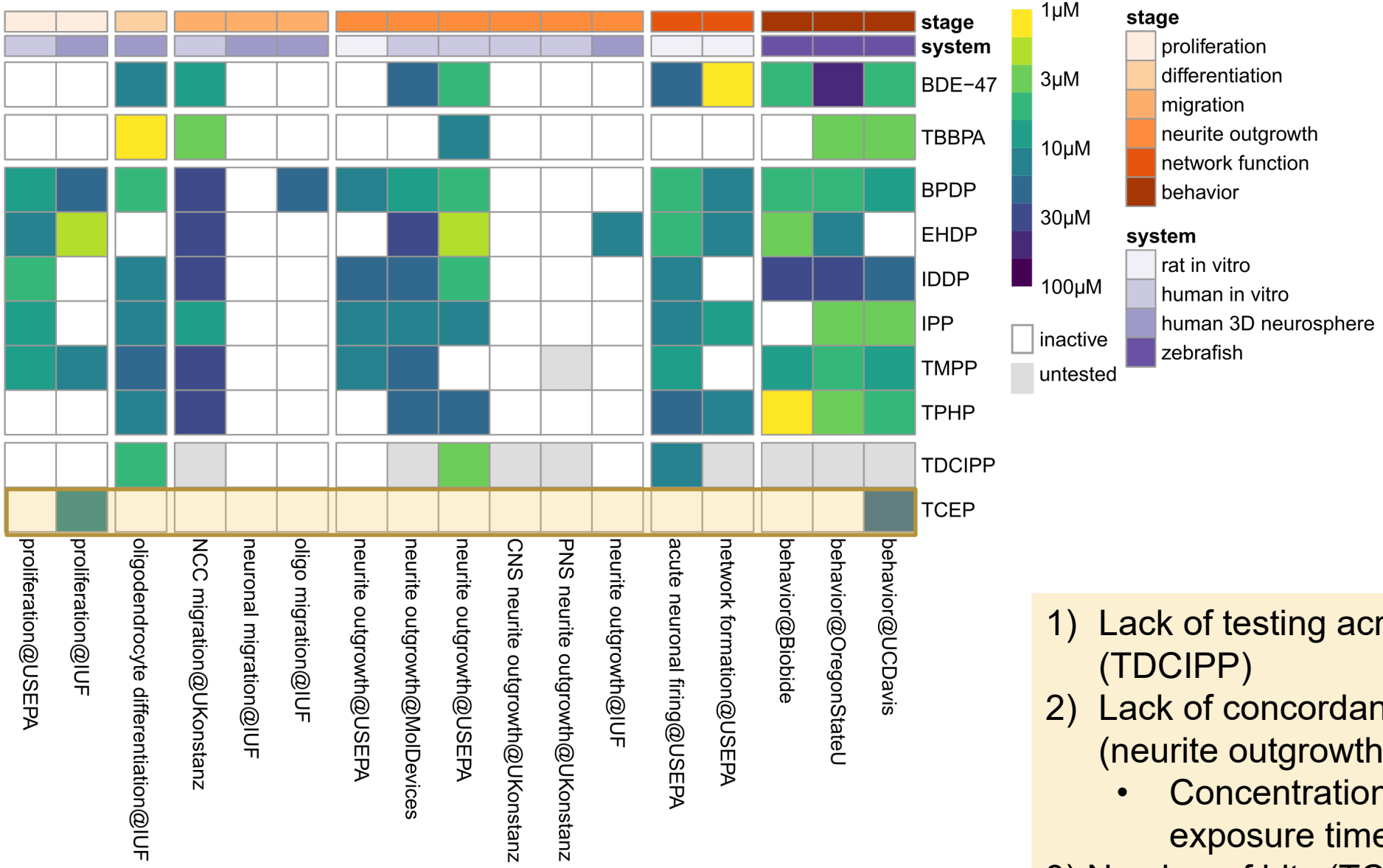
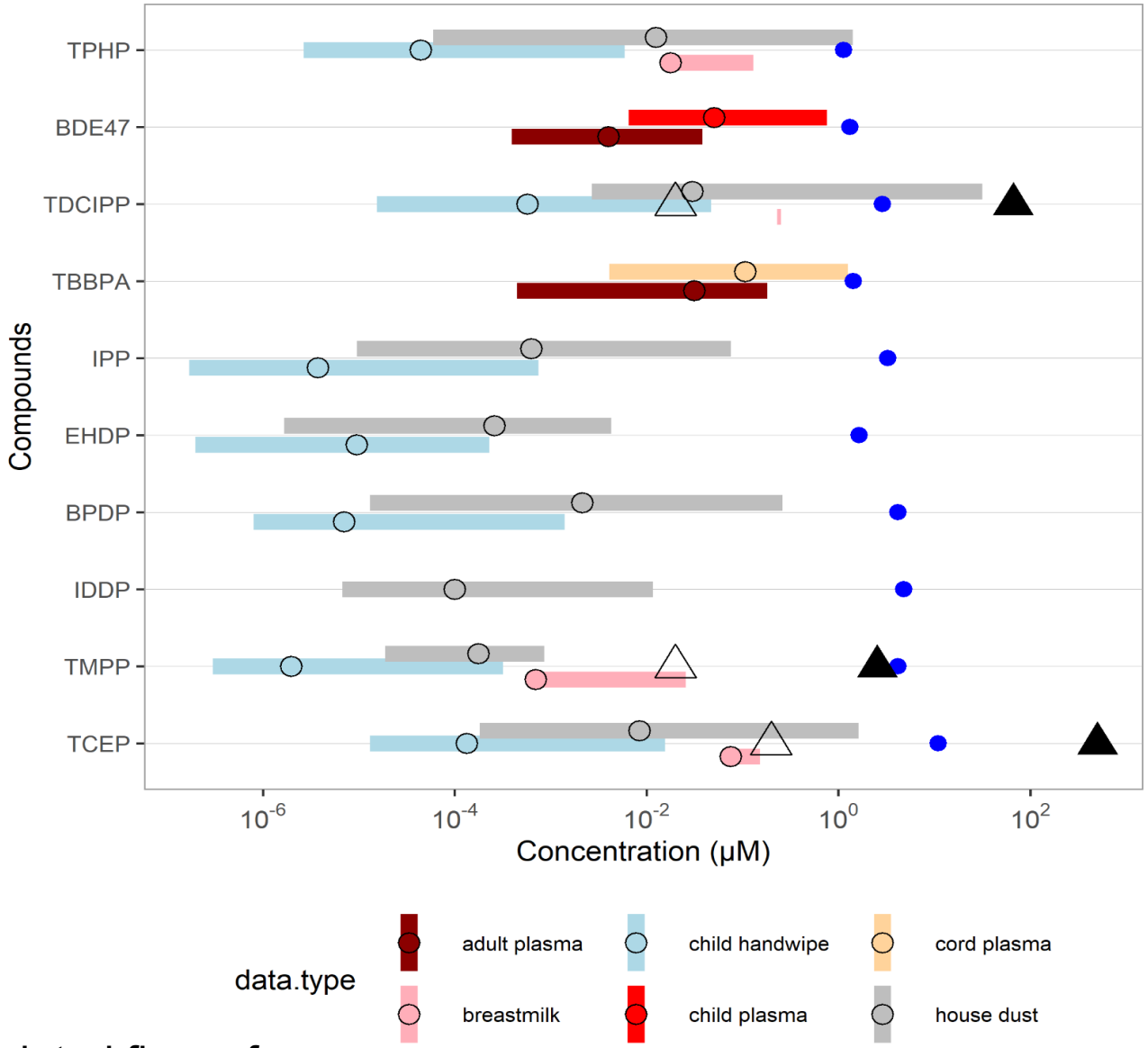


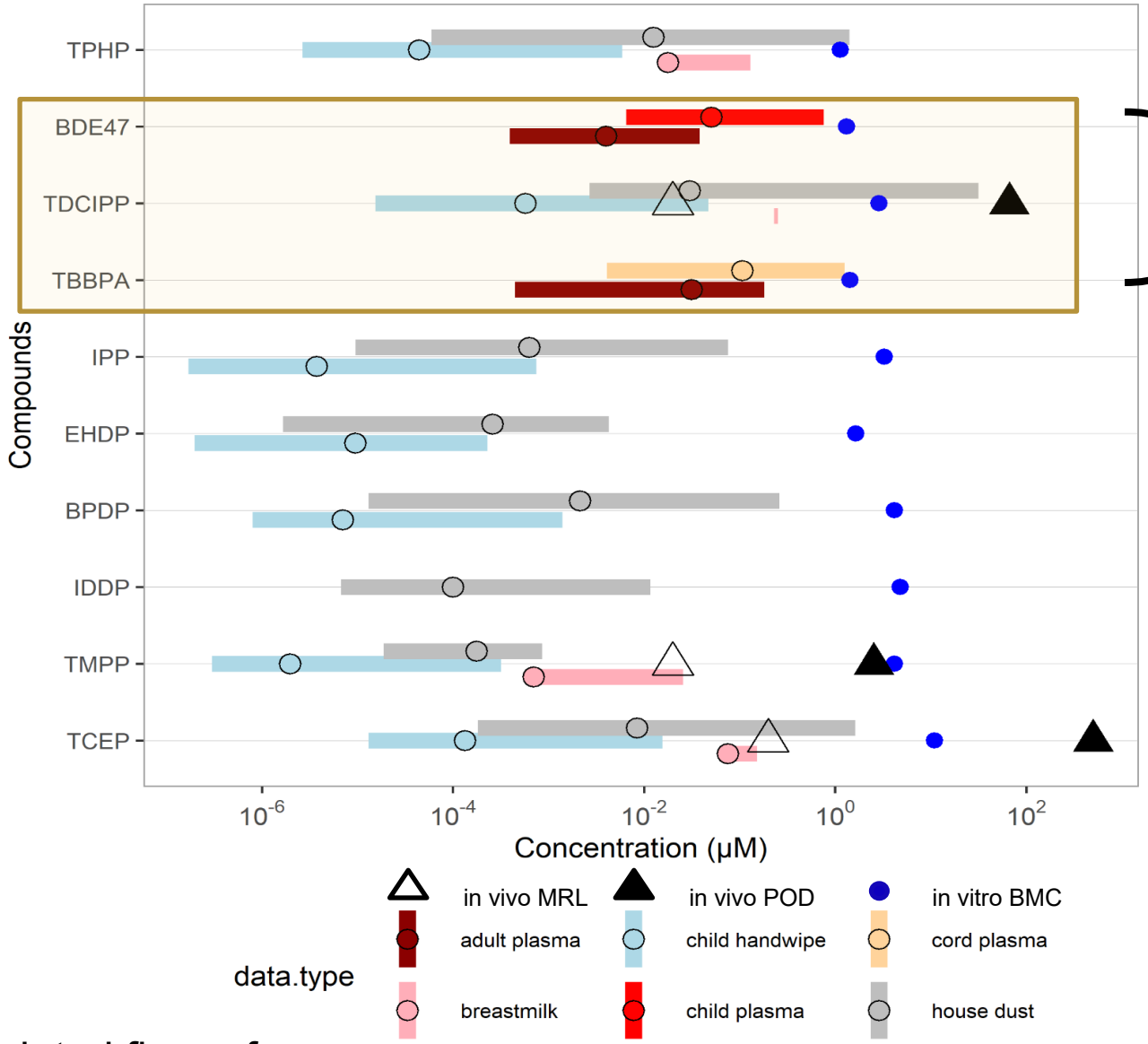
1) Lack of testing across assays (TDCIPP)
2) Lack of concordance within assays (neurite outgrowth, migration)
   - Concentrations, models, exposure time
3) Number of hits (TCEP)

Relevance to Human Exposures



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

Relevance to Human Exposures



Extensively used/ phased-out

Updated figure from
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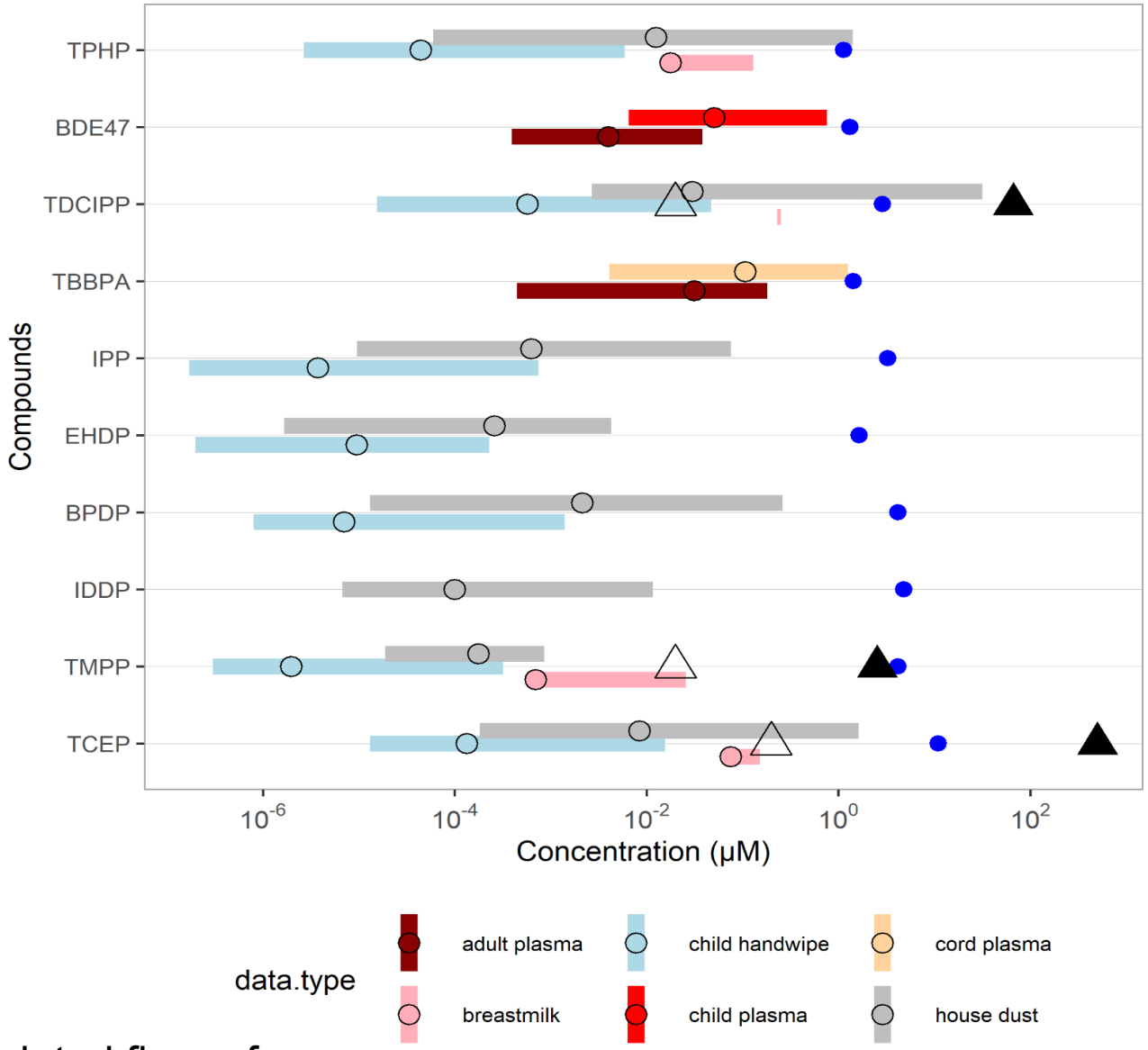
Relevance to Human Exposures



Novel Substitutes

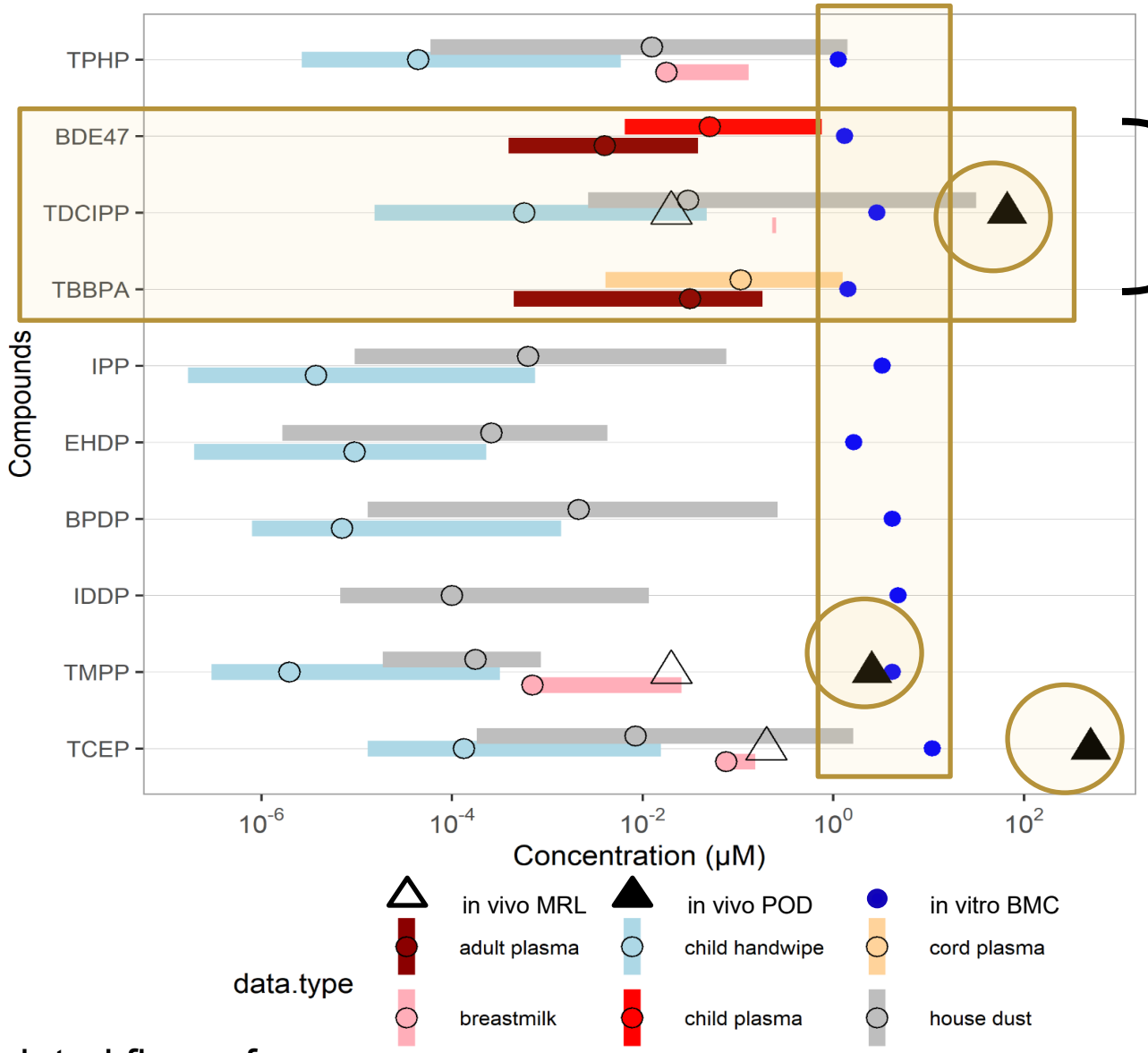
Extensively used/ phased-out

Novel Substitutes

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

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

Relevance to Human Exposures



Novel Substitutes

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Novel Substitutes

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)

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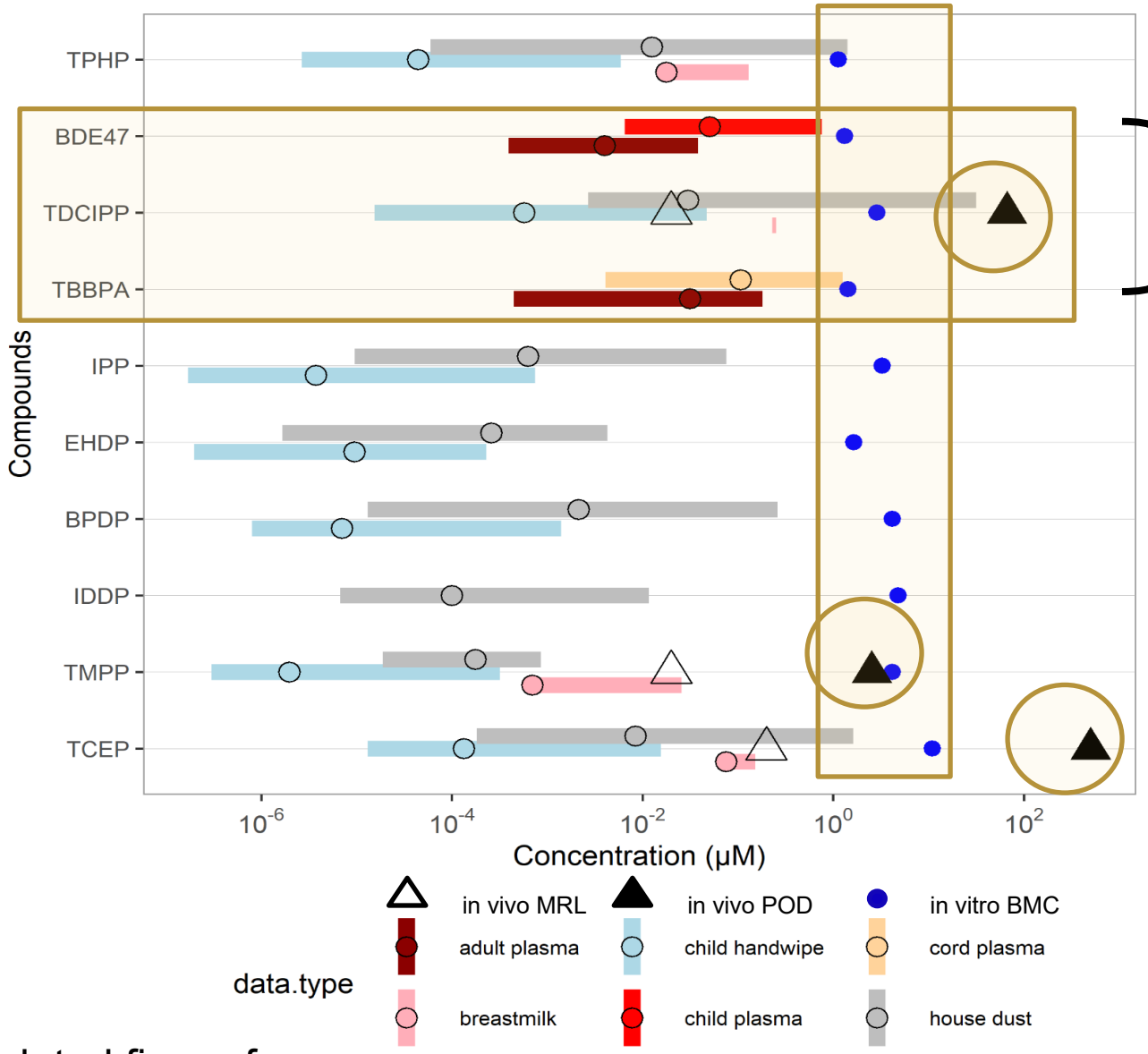
Relevance to Human Exposures



Novel Substitutes

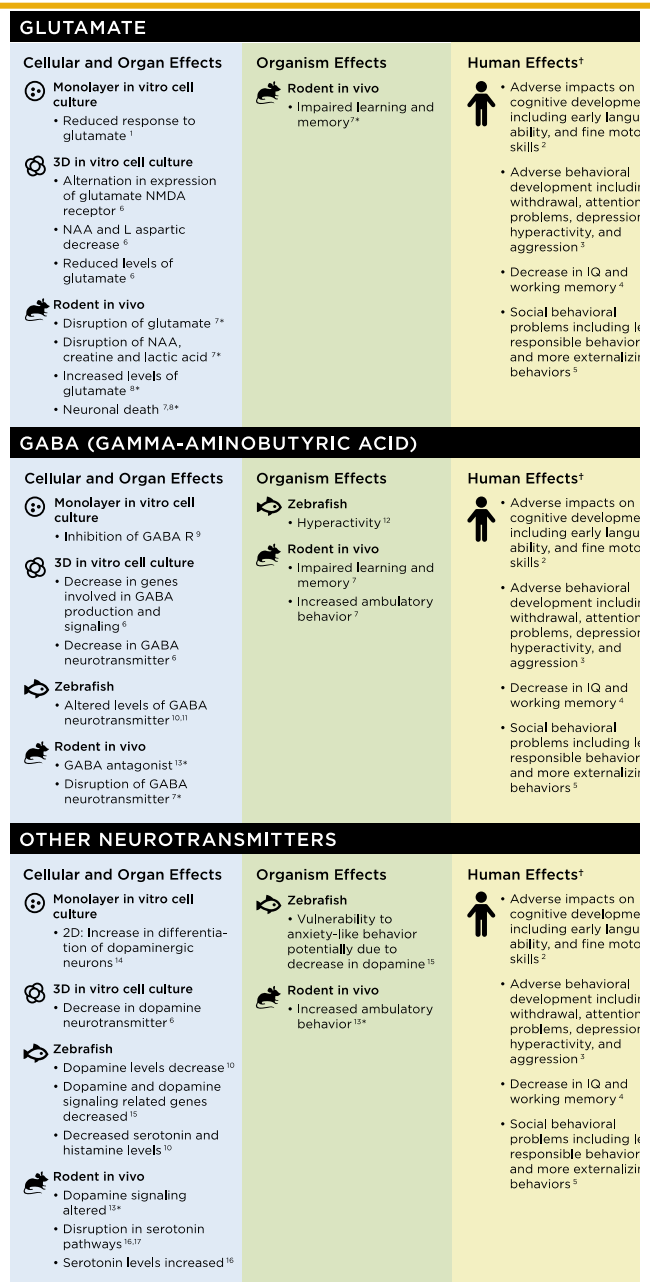
Extensively used/ phased-out

Novel Substitutes

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)

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

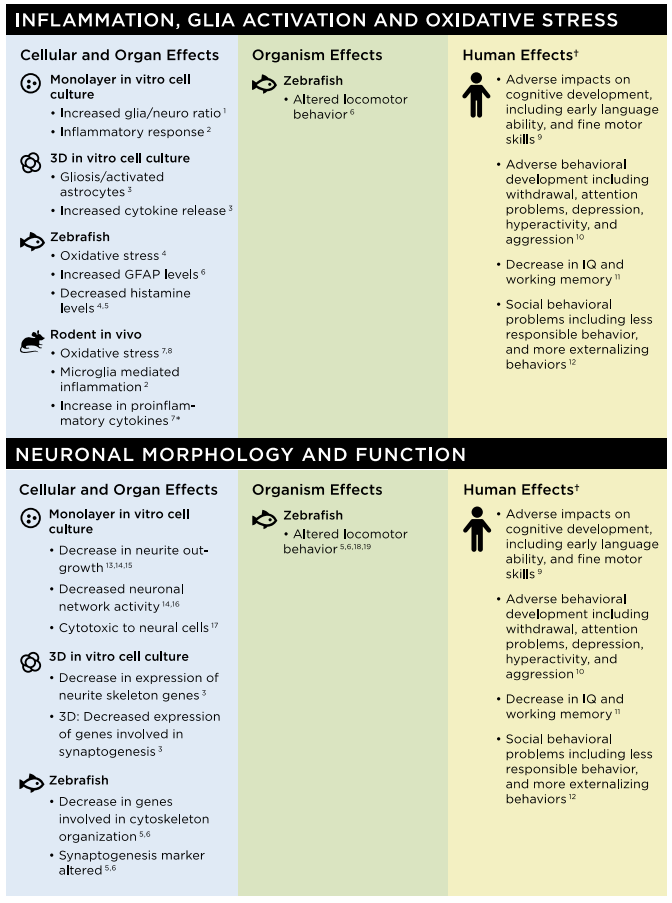
Consideration for further development of AOP



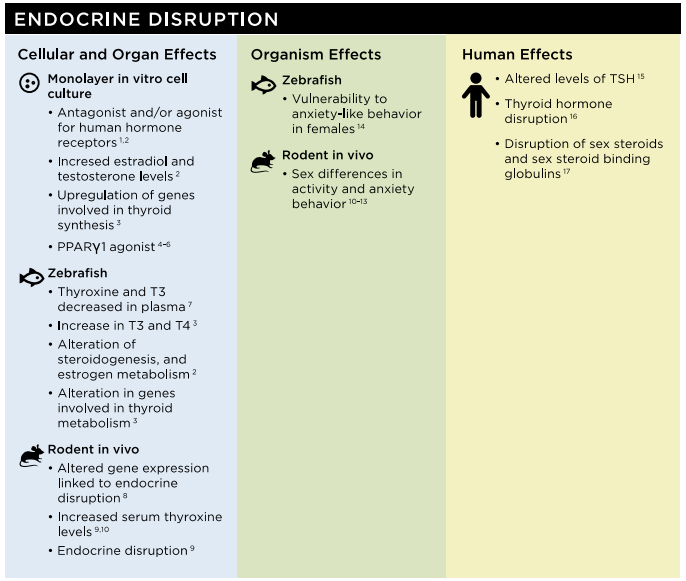
Beyond Cholinesterase Inhibition: Developmental Neurotoxicity of Organophosphate Ester Flame Retardants and Plasticizers

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. Stapleton[4]

Patisaul et al., 2021 EHE



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



Conclusions Case Study

- 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.

Future Directions

- 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

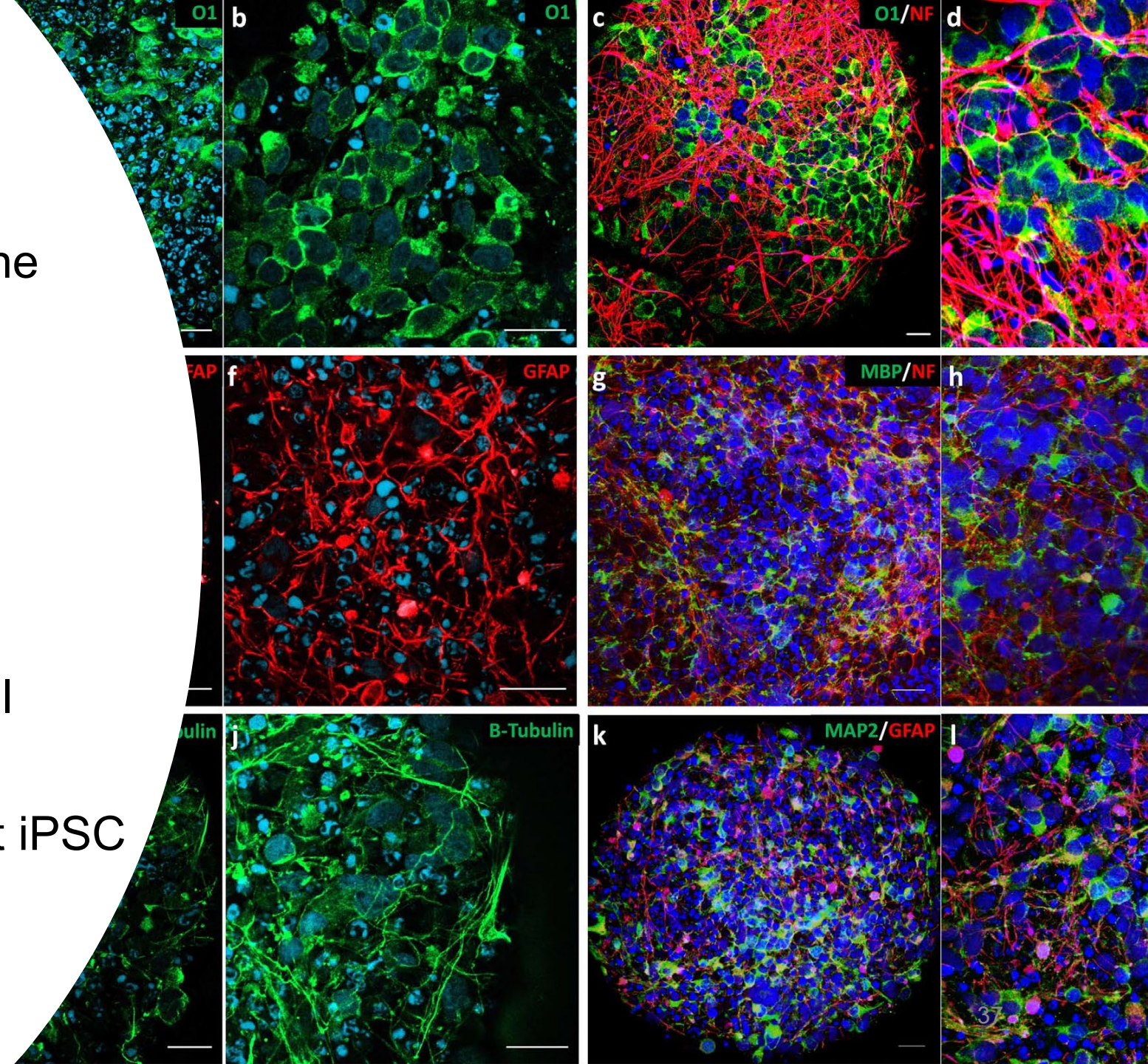
Microglia can be added

350-500 μm diameter

Reproducible in size and cell composition

Shown critical elements of neural development

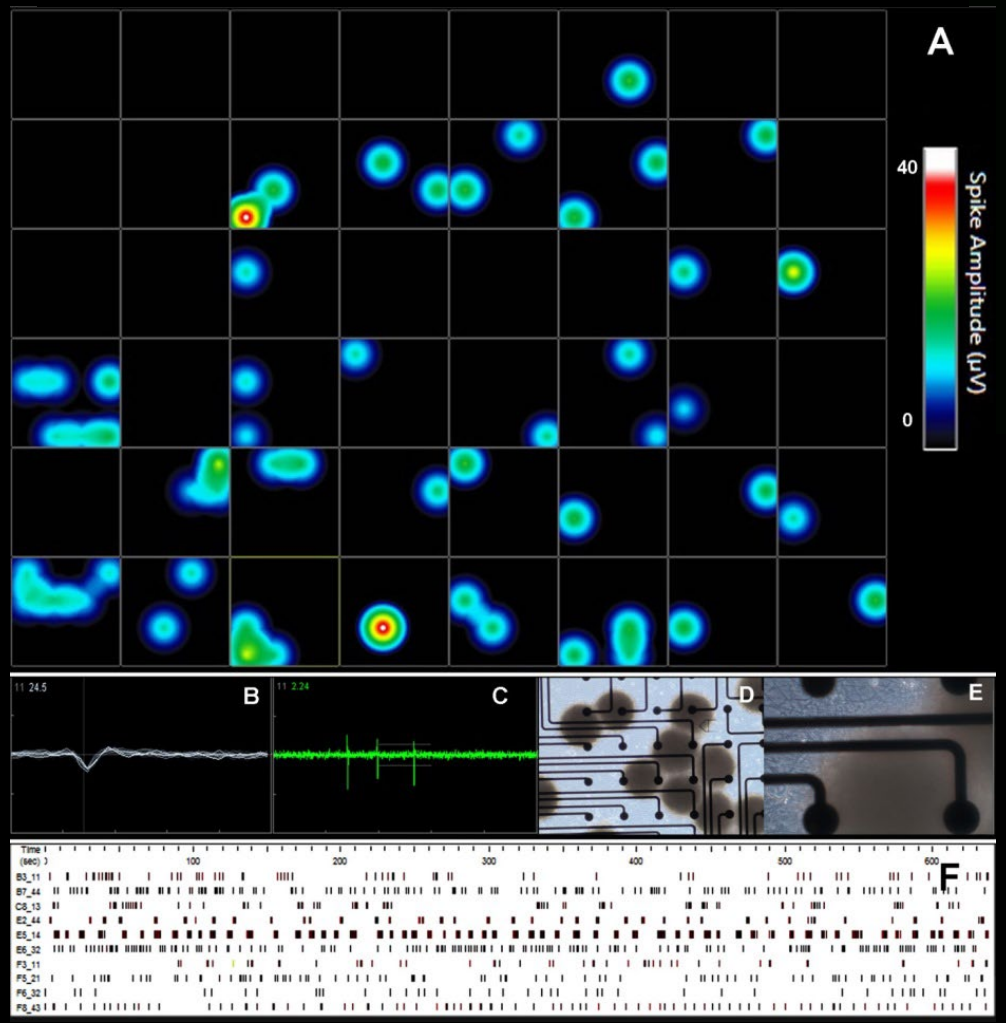
Genetic background from patient iPSC



Pamies et al., 2018, Tox. Appl. Pharmacol.

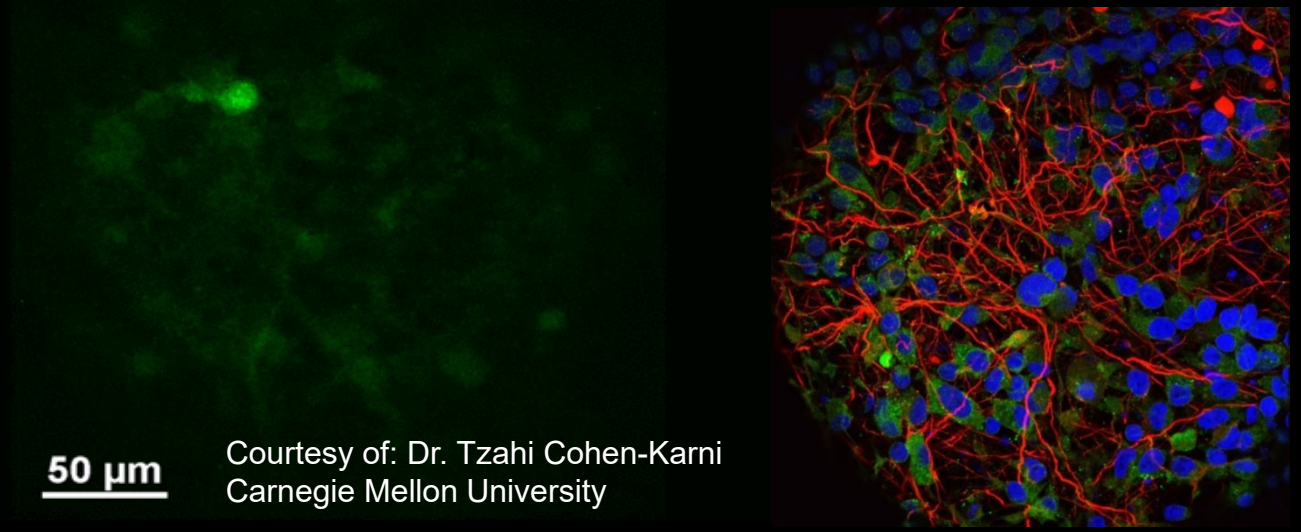
Functional neurons and glial cells

Micro-electrode arrays to measure spontaneous electrical activity

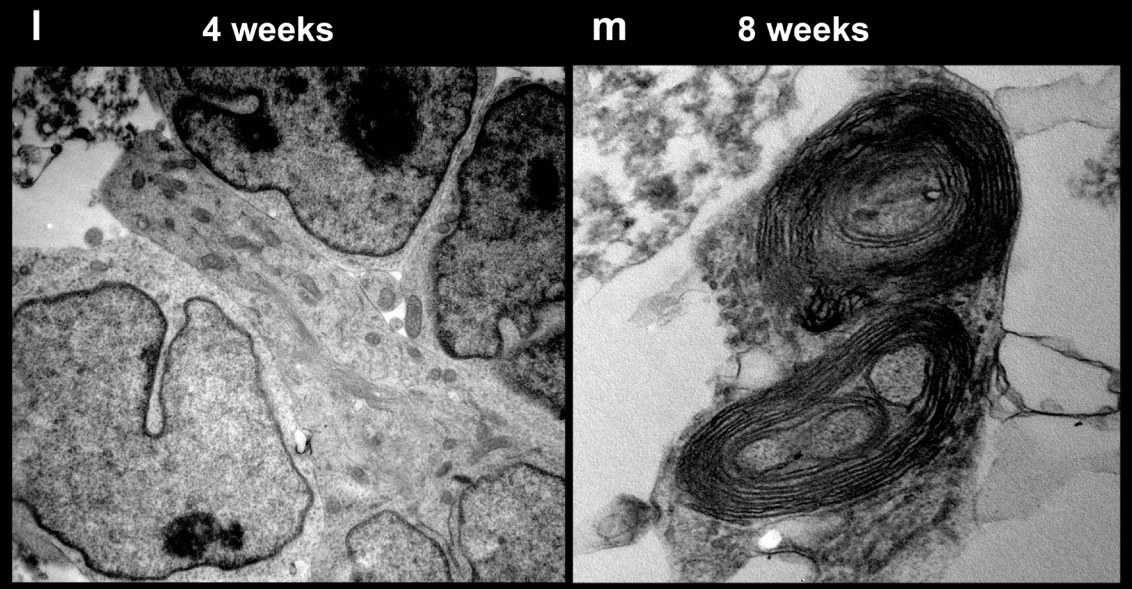


Pamies et al. 2017, ALTEX

Ca^{2+} signaling



Electron microscopy



Ongoing Work at DNTP

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

biobide
Your Zebrafish Partner

Assay development in vitro and in vivo

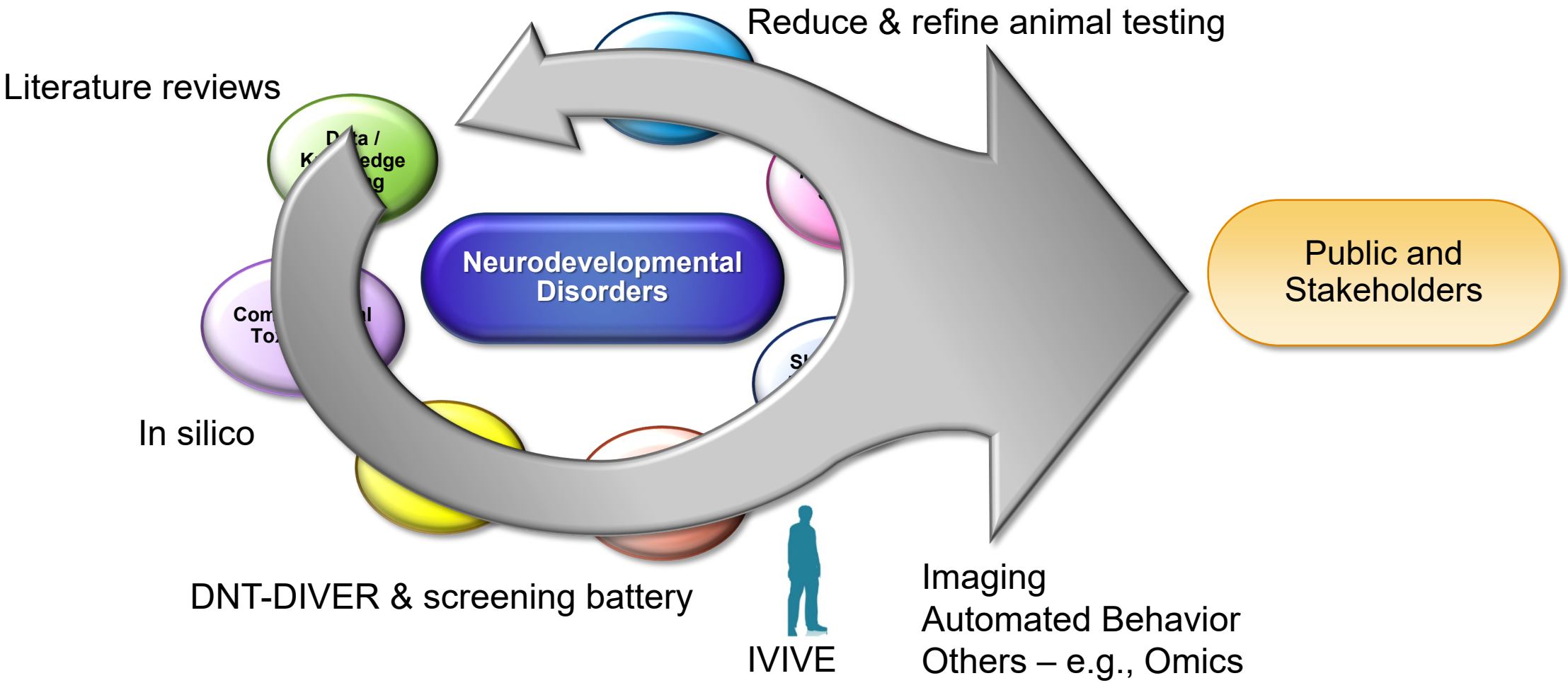
› Environ Health Perspect. 2021 Apr;129(4):47015. doi: 10.1289/EHP8314. Epub 2021 Apr 30.

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]

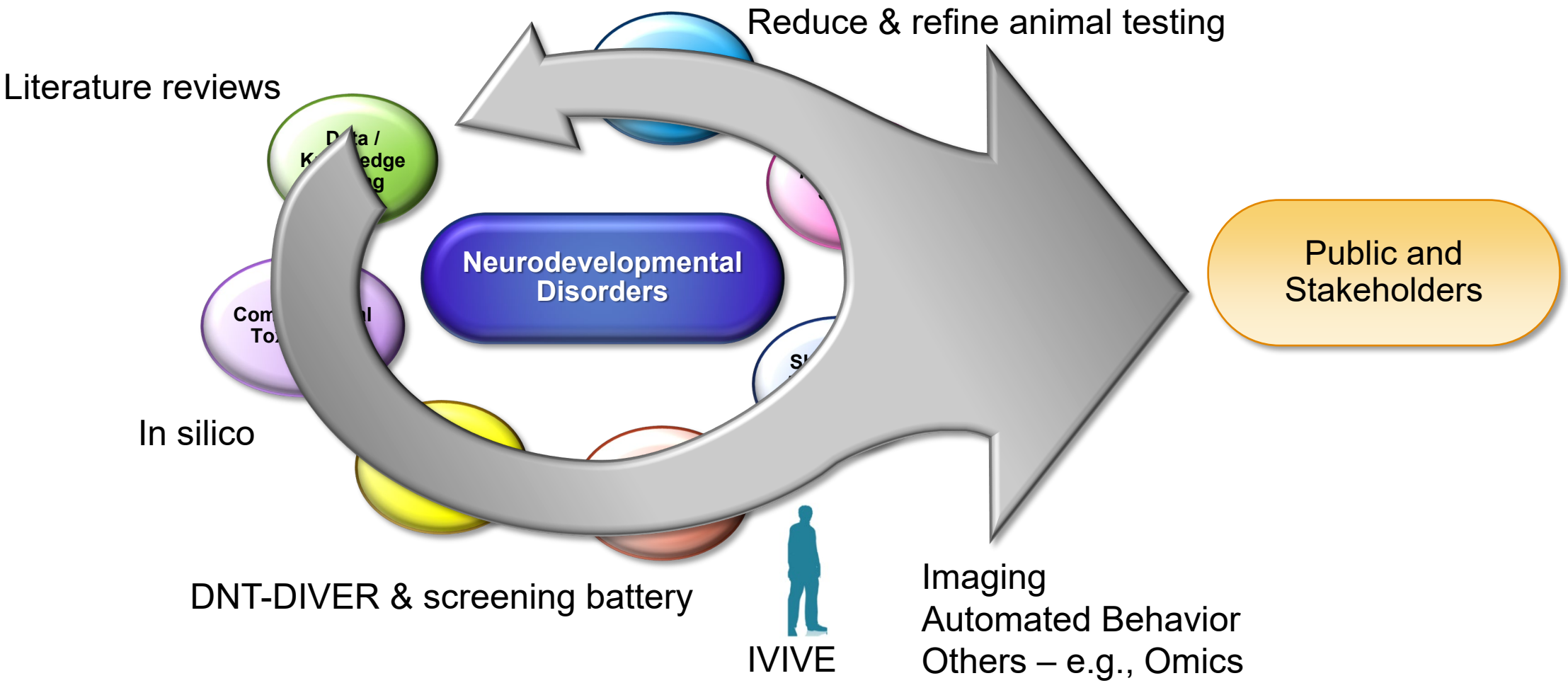
Linking mechanistic bioactivity to clinical end-points

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