Triangle Society for Neuroscience Meeting

Development and Use of New Approach Methodologies for Developmental Neurotoxicity Hazard.

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Disclosure Statement

This work has been funded by the US. Environmental Protection Agency. I have no conflicts to declare.

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Overview

I. Background- why we need New Approach Methodologies (NAMs) for developmental neurotoxicity (DNT).

II. Approach to NAMs development for DNT.

III. Overview of the EPA DNT in vitro battery (DNT-IVB).

IV. Case-studies of NAMs use for regulatory decision-making and their impact.

A basic toxicological premise: **Risk = Hazard x Exposure**

How does the EPA regulate chemicals?

Toxic Substances Control Act (TSCA)

All New Chemicals
>60-80K "Grandfathered" Chemicals ("existing" chemicals)

Available Data
90 Day Premanufacture Notice

"Data Poor"- little or nothing may be known about toxicity hazard

Intended to Kill Something

Federal Insecticide, Fungicide and Rodenticide Act (FIFRA)

All "Pesticides"

Required **Guideline Studies**
Health and Environmental Effects

Data Rich- Toxicity hazard is well characterized

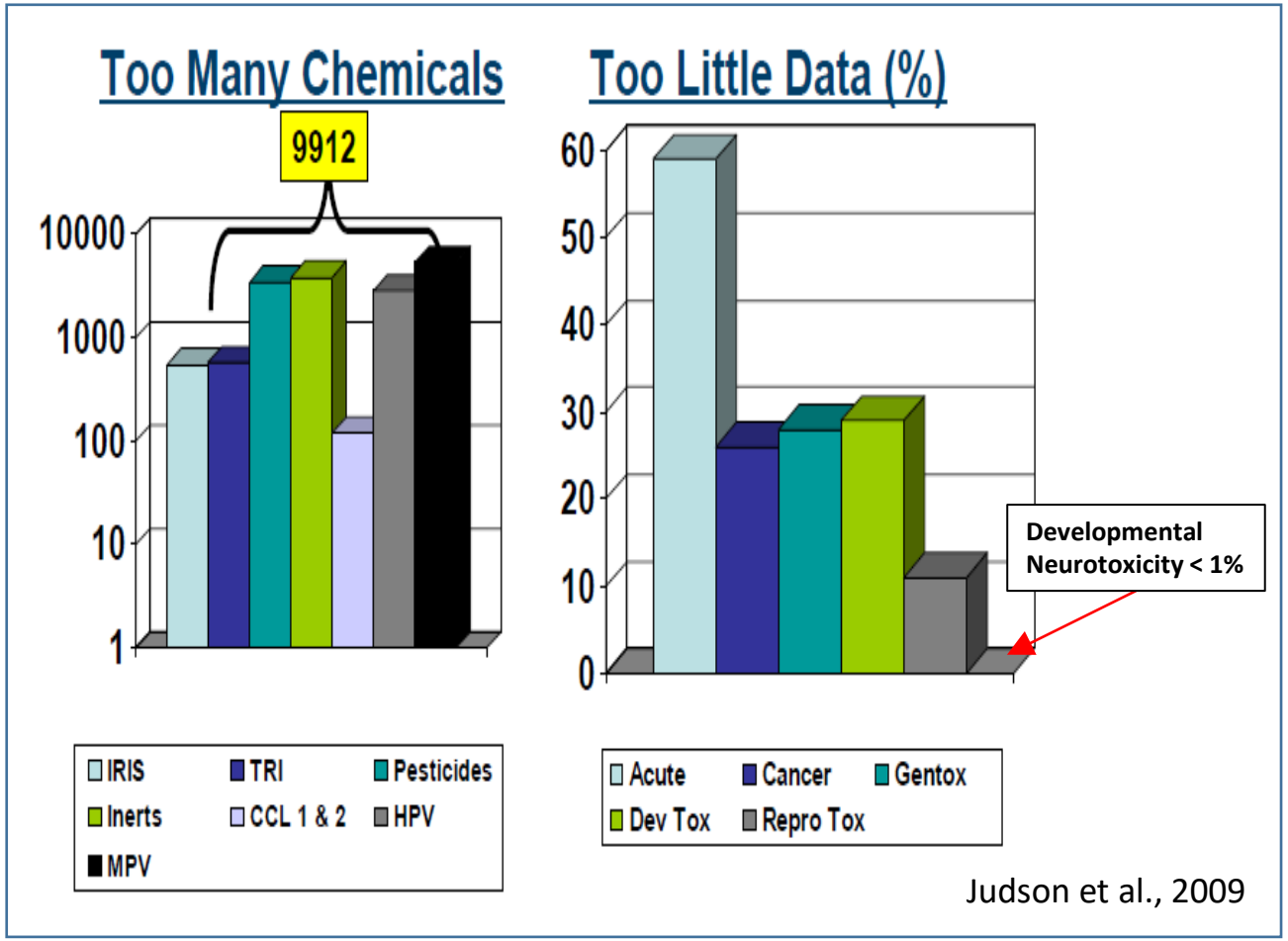
Lautenberg Chemical Safety Act 2016

- **Mandatory requirement for EPA to evaluate existing chemicals with clear and enforceable deadlines;**
- **Risk-based chemical assessments;**
- **Increased public transparency for chemical information;**
- **Consistent source of funding for EPA to carry out the responsibilities under the new law.**
- **Must consider risks to susceptible and highly exposed populations**
- **Directs EPA to utilize alternatives to animals**

Food Quality Protection Act of 1996

- **Mandates an extra 10x safety factor for children/infants**
- **Mandates Assessment of Cumulative Risk to Pesticides with the same mode of action**

Many Chemicals Lack Developmental Neurotoxicity (DNT) Hazard Data



Judson et al., 2009

Public Concern

Reports of the potential involvement of environmental chemicals in increased rates of neurodevelopmental disease contributed to increasing public concern about DNT hazard of chemicals

Current testing is too slow; "Guideline" DNT:

- Not Required under TSCA, triggered under FIFRA
- 1 chemical= $1M cost; 2 yr; 1000 animals
- At current pace, ~150 chemicals in 20+ yrs
- Not often used (~25%) for point of departure values for risk assessment*

The absence of DNT hazard data on chemicals impedes consideration of this adverse outcome in environmental decision-making.

*Raffaele et al. The use of **developmental neurotoxicity** data in pesticide risk assessments. Neurotoxicol Teratol. 2010 Sep-Oct;32(5):563-72.

Requirements of EPA 870.6300 (OECD TG 426/443)

- 6 Pregnant females/dose (20 litters/dose recommended)
- 10 pups/litter (5 male/5 female)
- Minimum 3 doses + control
- Dosing period GD6-PND10
- Assessments on PND 4, 11, 21, 35, 45, 60

- Signs of Maternal Toxicity
- Developmental landmarks
- Brain/body weights (4, 11, 17, 21 PND)
- Motor activity (13, 17, 21, 60 PND)
- Auditory Startle (weaning, PND 60)
- Learning and memory (weaning, PND 60)
- Neuropathology (PND 11 and termination)
  - Major brain regions

While this study provides a broad coverage of structural and behavioral endpoints, it does not provide any information on the underlying biology that has been impacted; no data from human models-

This requires extrapolating results to humans and introduces uncertainty.

https://beta.regulations.gov/document/EPA-HQ-OPPT-2009-0156-0042
https://www.oecd-ilibrary.org/environment/test-no-426-developmental-neurotoxicity-study_9789264067394-en
https://www.oecd.org/chemicalsafety/test-no-443-extended-one-generation-reproductive-toxicity-study-9789264185371-en.htm

Solution to the lack of DNT Data

Faster, inexpensive and predictive methods are needed to detect and characterize compounds with developmental neurotoxicity hazard

- Develop high throughput, *in vitro* assays,
- Characterize chemicals for developmental neurotoxicity hazard
- Use human models whenever possible

Data from these New Approach Methodologies (NAMs) can provide information for decision-making

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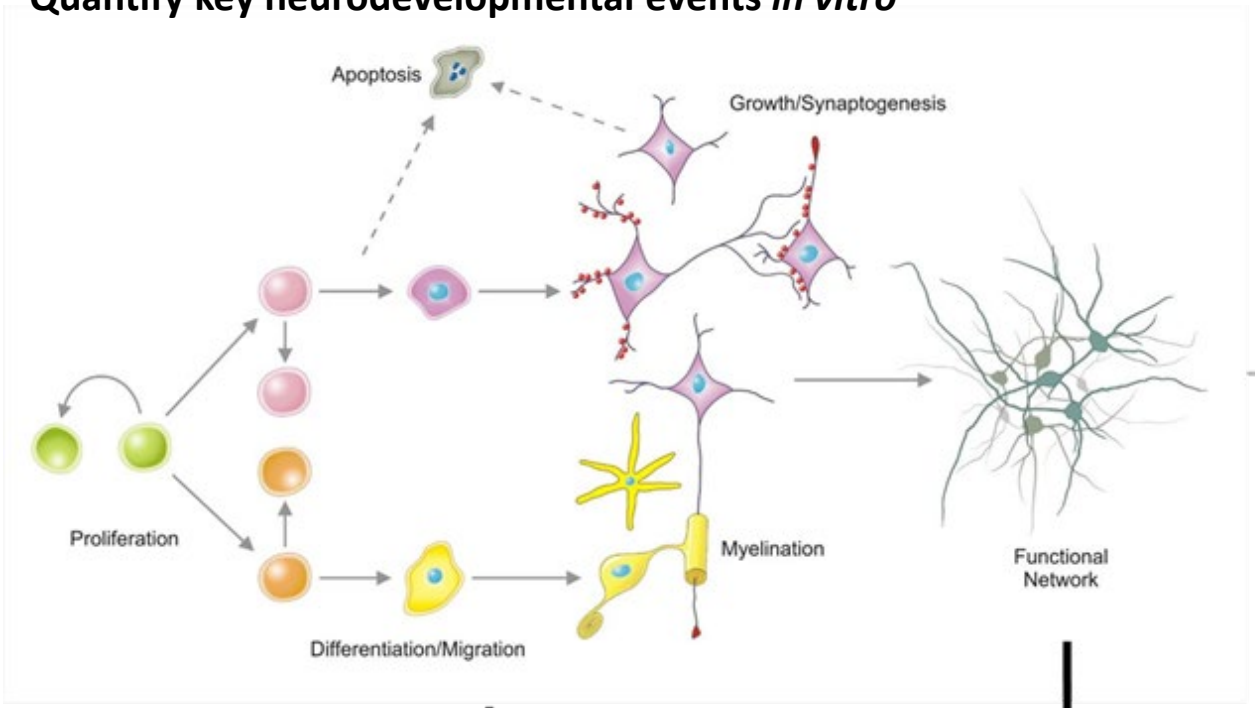
Challenges to Development of DNT NAMs

- Central nervous system development is complex
  - Multiple potential targets
  - Time-dependent processes
  - Spatially-dependent processes
- Which target? Where? When?

Therefore, focus research on *key neurodevelopmental processes*

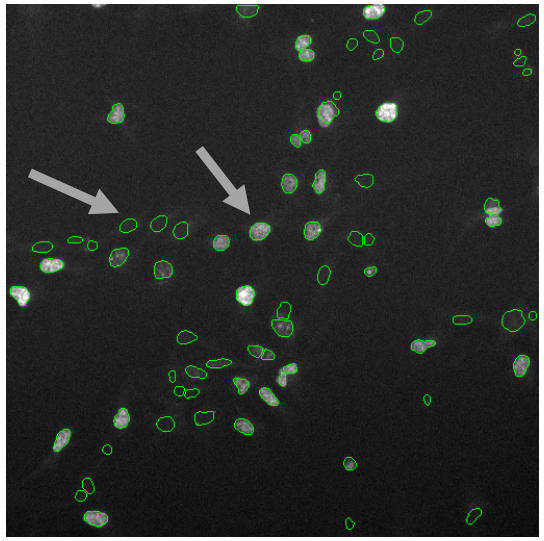
Phenotypic Screening for DNT Hazard

Quantify key neurodevelopmental events *in vitro*

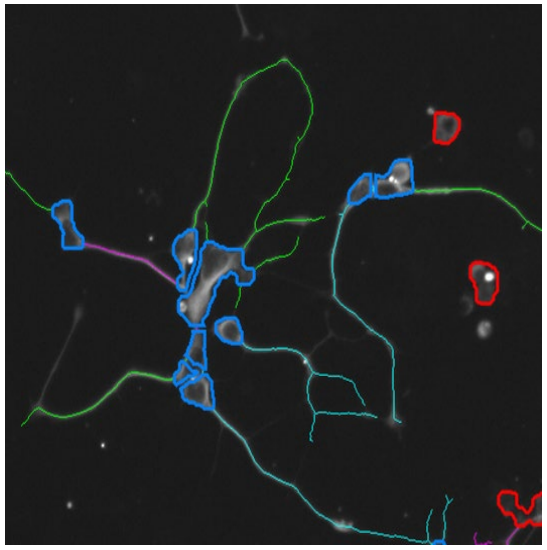


EPA DNT NAM Assays

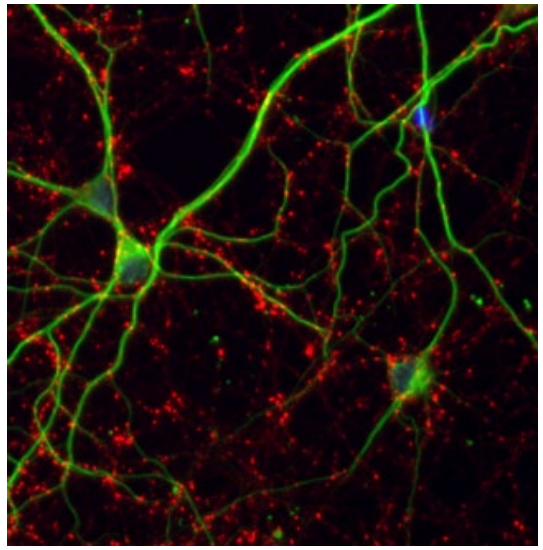
Proliferation



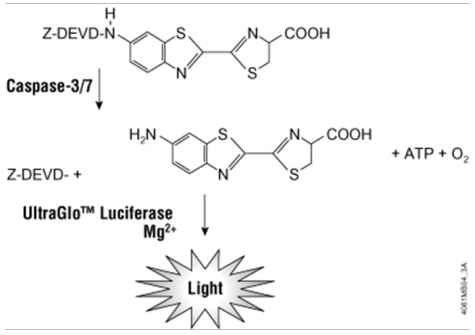
Neurite Outgrowth



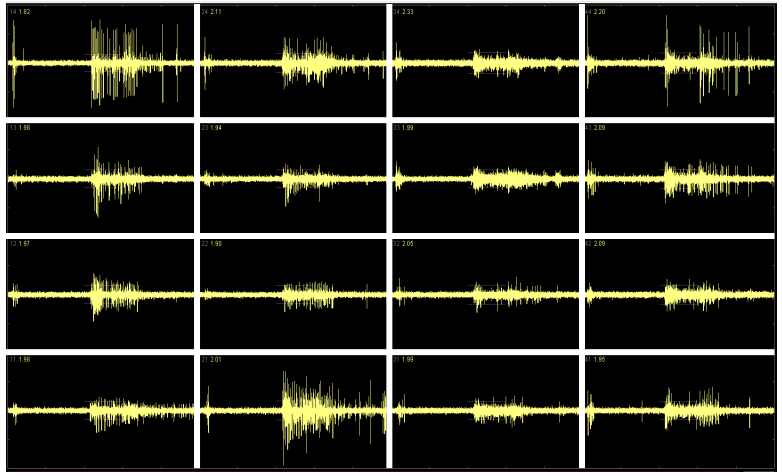
Synaptogenesis



Apoptosis

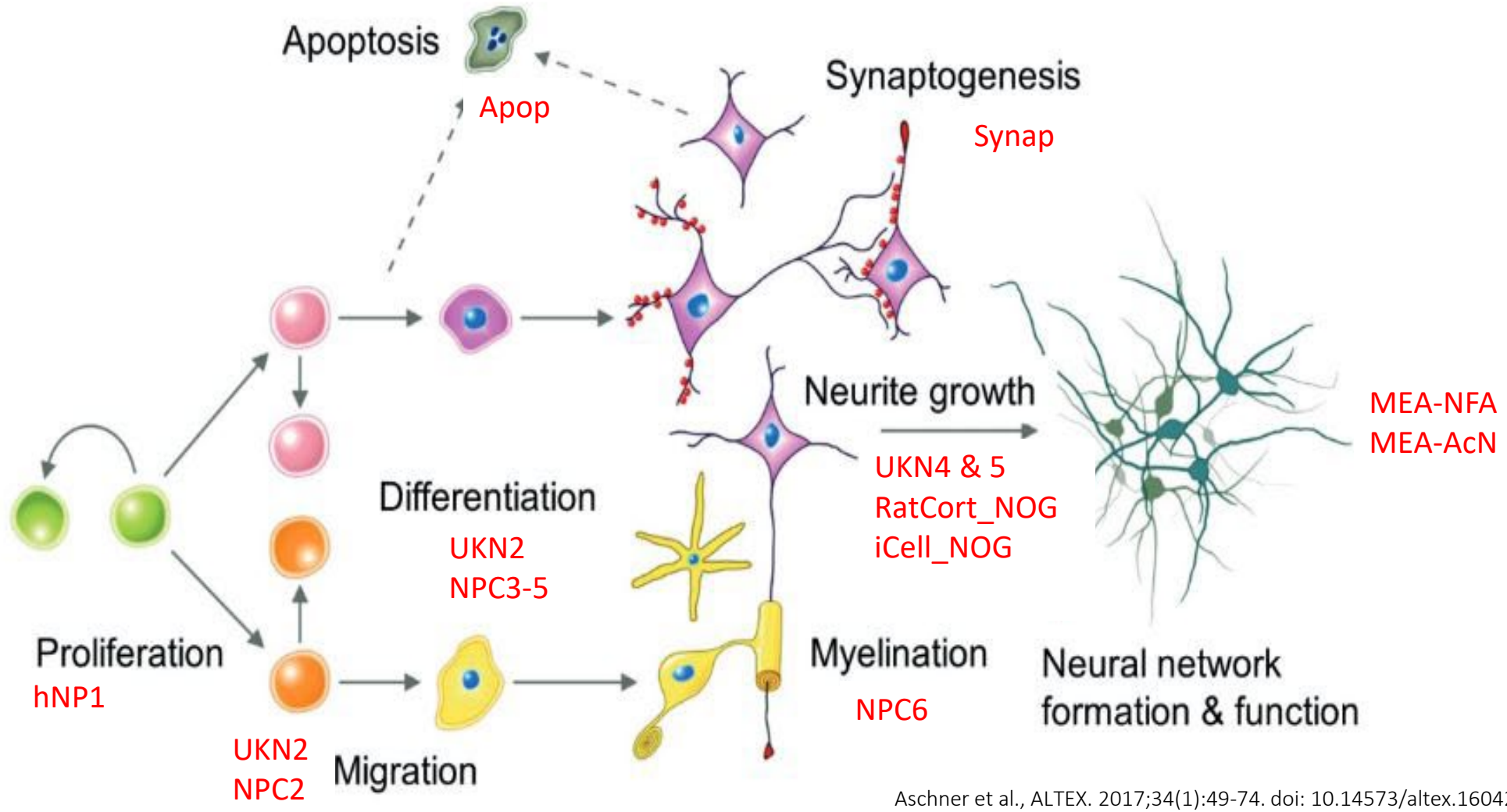


luminescence



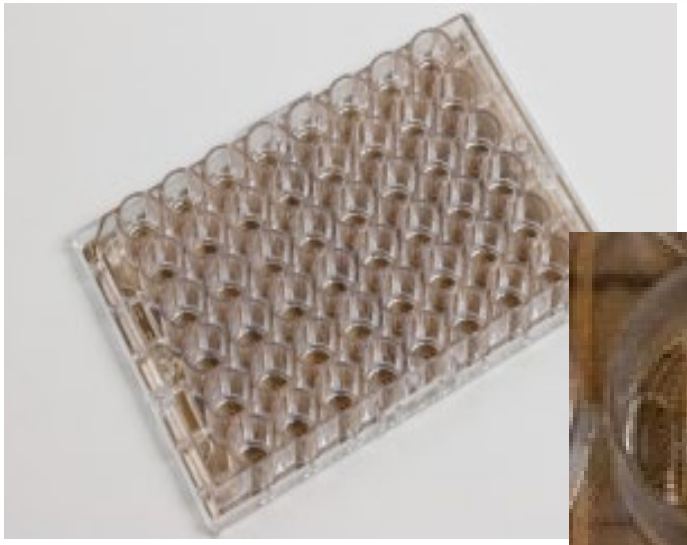
Network Formation Assay (NFA)

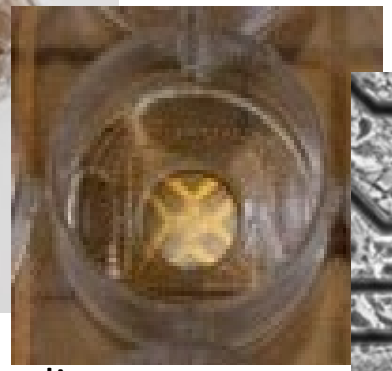
DNT NAMs Coverage of Neurodevelopmental Processes

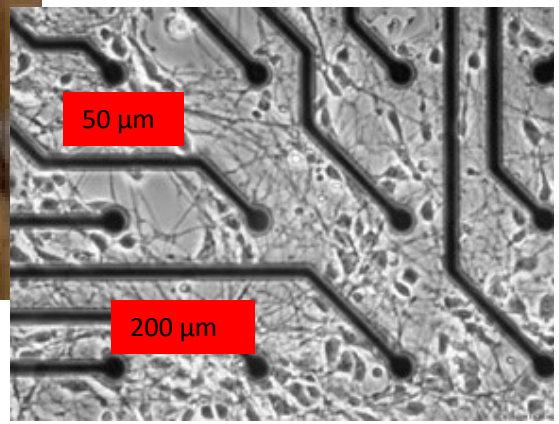


Aschner et al., ALTEX. 2017;34(1):49-74. doi: 10.14573/altex.1604201

Measuring Network Formation on Microelectrode Arrays







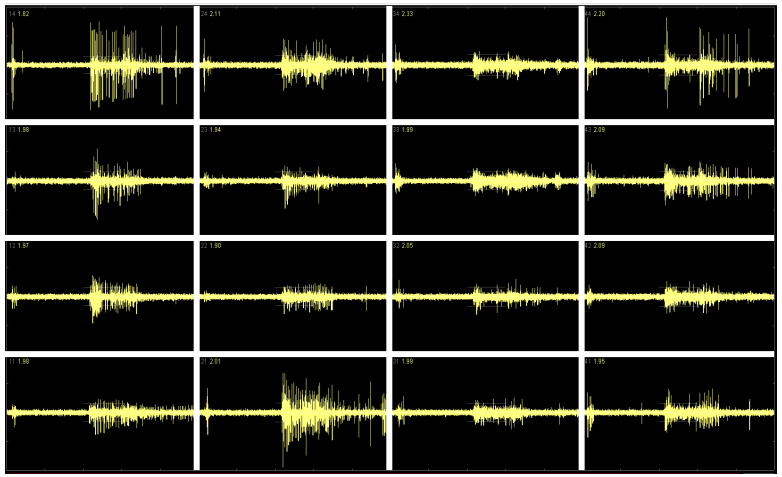
Microelectrode Array (MEA) Recording

- Planar microelectrodes are non-invasive
- Records electrical activity of any tissue type
- Repeated recordings from same sample

The electrical activity recorded by MEAs are the biological underpinnings of EEG recordings.

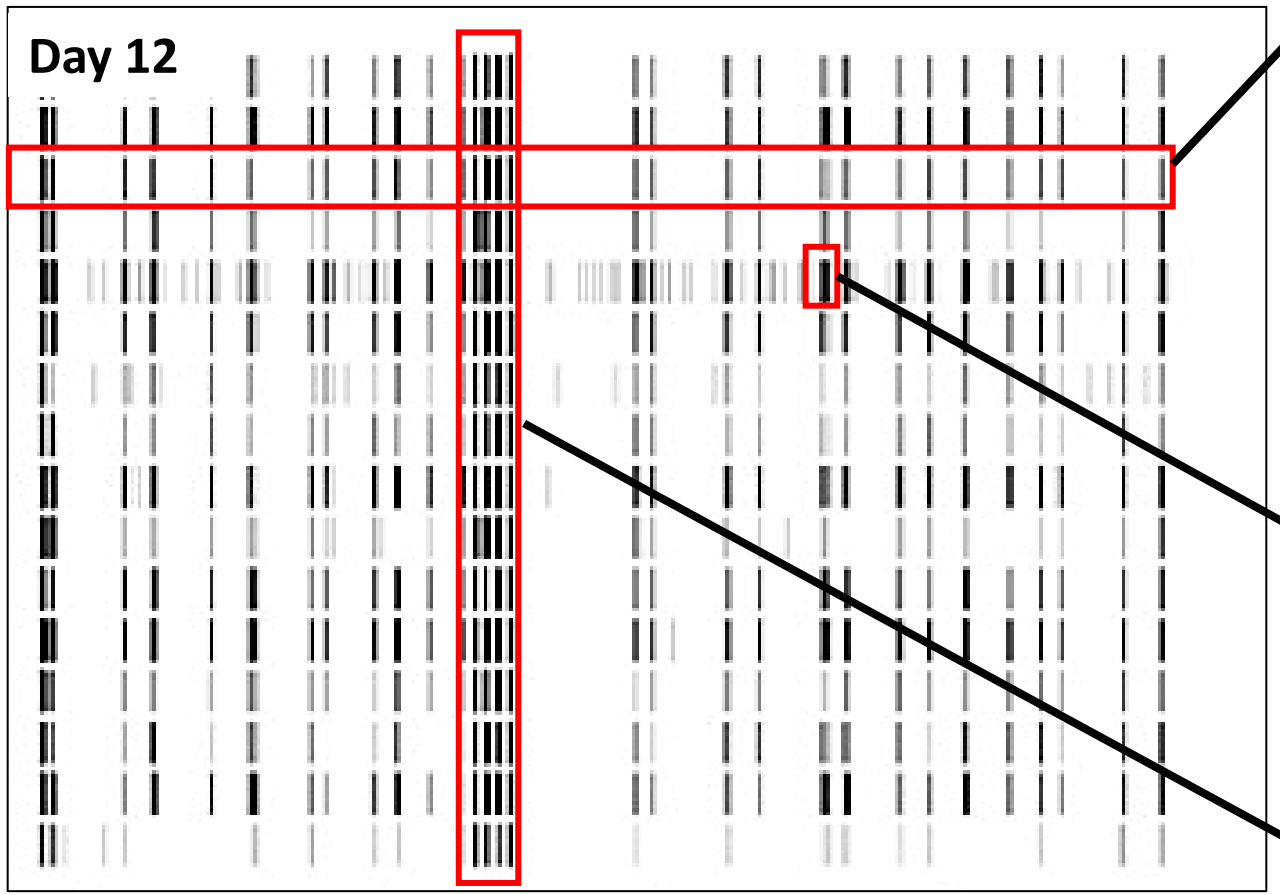
"Brain-on-a-Chip": Complex 2D model

- Rat cortical neural networks
- Contains neurons & glia cells
- Spontaneous activity
- Develops rapidly in vitro
- Follow network development over time
- Integrates activity of multiple processes



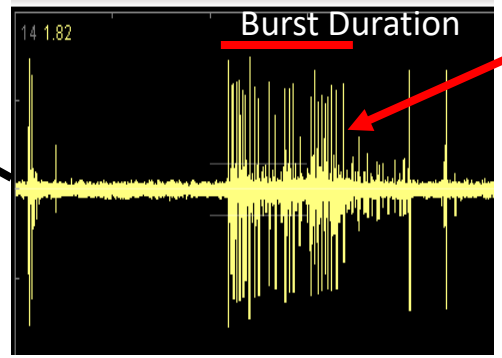
A snapshot in time of neural network activity in one well. Each box represents the electrical activity of neurons on 1 electrode in the array.

MEAs Measure Multiple Characteristics of Network Formation



General Activity- overall rate of firing or bursting; measured on each electrode and averaged across the well.

Bursting Structure- the length and number of events in a burst; measured on each electrode and averaged across the well.

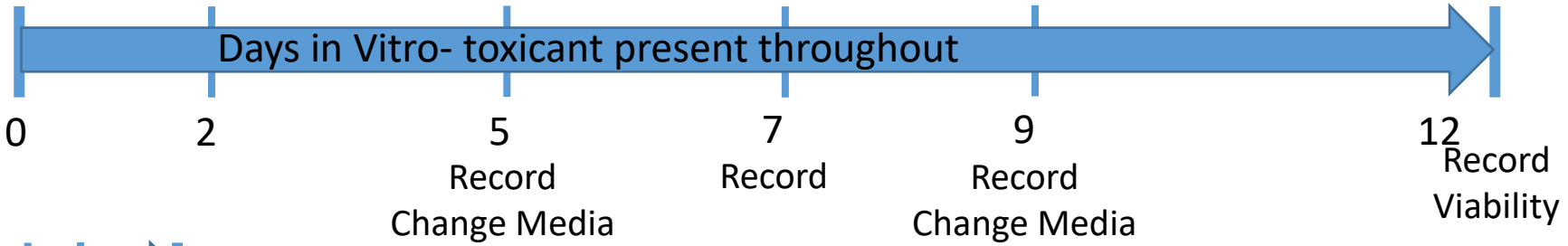


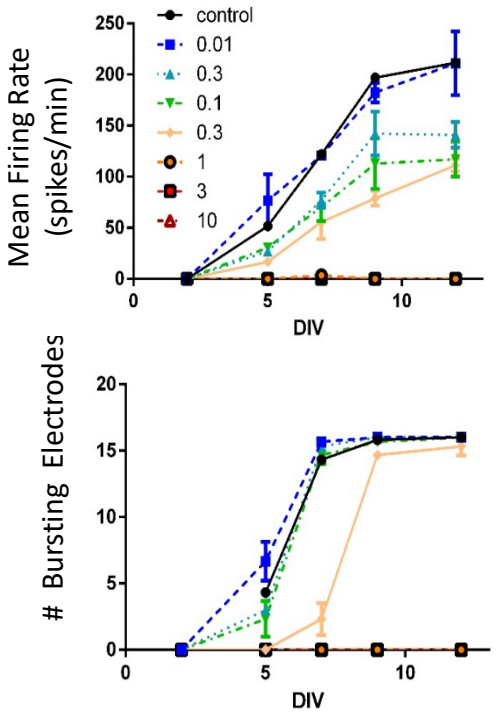
Number of Action Potential "Spikes"/burst

Connectivity- Communication of information across electrodes (Correlation coefficients, Network Spikes, Mutual Information); averaged for the well.

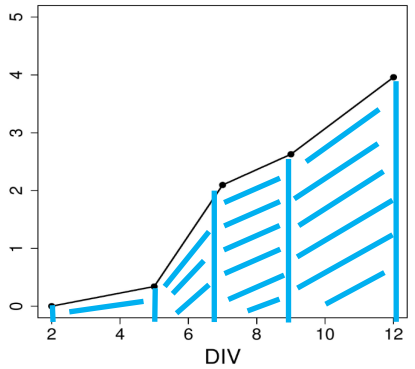
In the Network Formation Assay (NFA), 19 endpoints describing network activity (17) and cytotoxicity (2) are measured over 12 days in vitro. These can increase or decrease following chemical exposure.

The Network Formation Assay



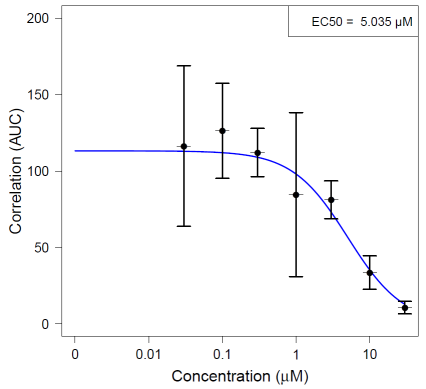


Determine Area Under the Curve for each dose (8) and parameter (17)

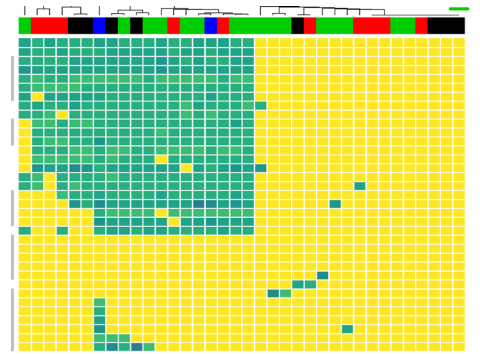


Tcpl*

Determine concentration-response



Generate comparisons of potency for many chemicals and endpoints



***tcpl** =toxcast pipeline

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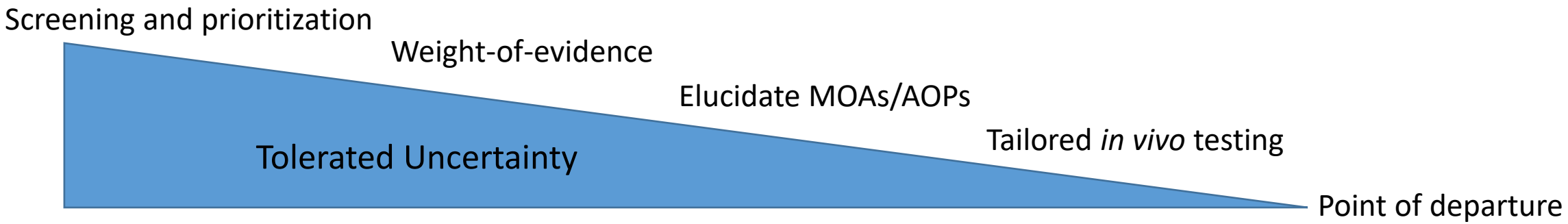
Use of NAMs in Risk Assessment- What's your problem?

Problem Formulation is critical to use of NAMs

- What decision needs to be made?
  - Screening and Prioritization
  - Aid in deciding if additional studies are/aren't needed
  - Aid in interpretation of *in vivo* observations
  - Leverage understanding of underlying biological processes
- What types of data are needed to make that decision?
  - Bioactivity?
  - Exposure?
  - Kinetic/Metabolism?
- How much uncertainty in the data is acceptable?



Examples of the use of DNT NAMs at EPA

I. **Screening Level information**
   - Any hazard data for DNT is lacking. Is there a concern for DNT?

II. **Weight of Evidence (WoE) approaches**
   - Are additional studies needed?
   - Can the Agency grant a waiver for a guideline DNT study?

Example #1: Screening Level Information for PFAS Compounds

Problem: Perfluoroalkyl substances have recently been identified as environmental contaminants with significant human exposure. Little toxicological information is available for these compounds.

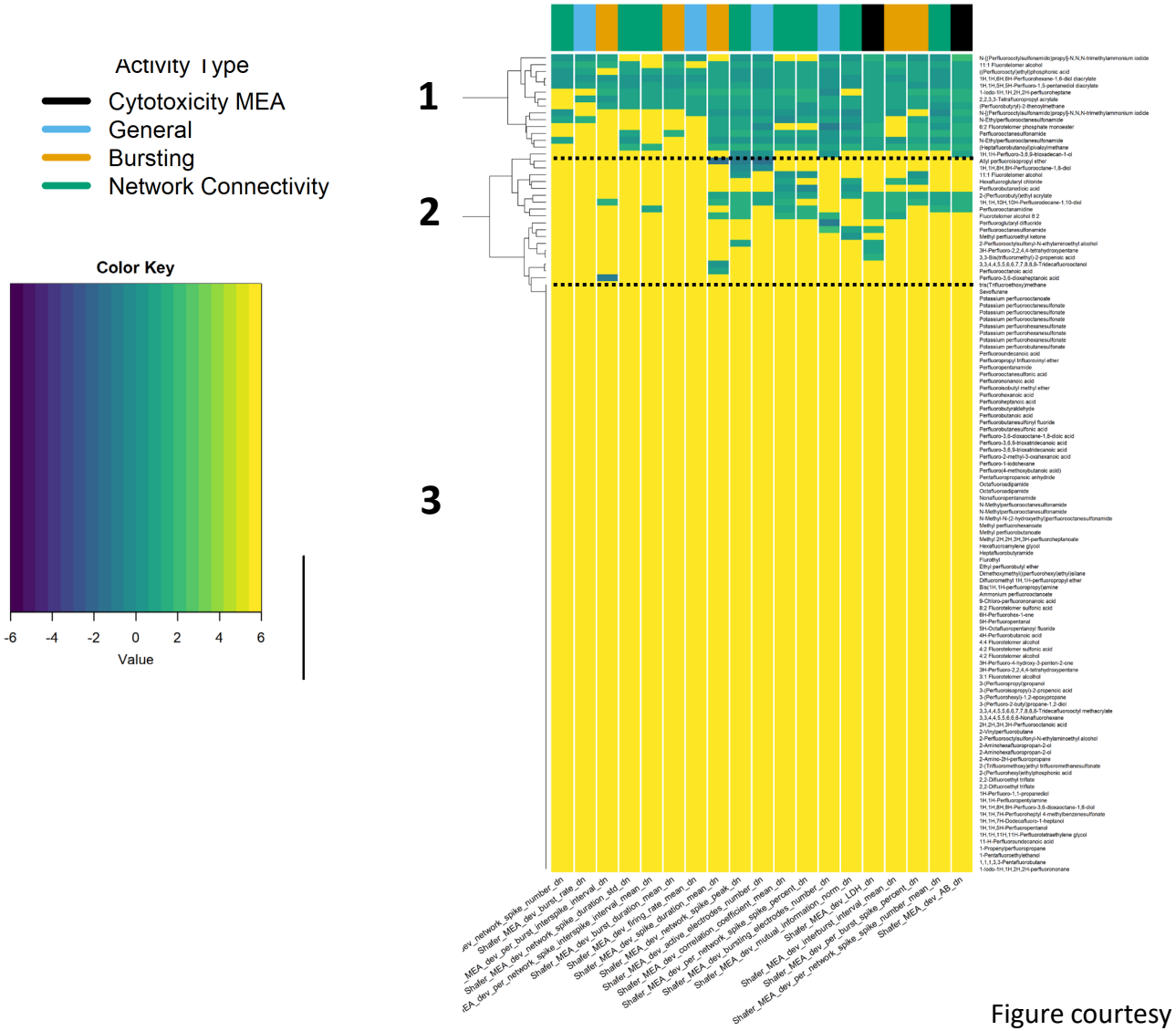
- Structurally diverse
- With the exception of a few specific congeners, little toxicological information
- Evidence of DNT is ambiguous,
  - epidemiological studies reports are equivocal
  - neurodevelopmental effects associated with exposure to PFAS in rodent and other animal studies

Assembled a PFAS Chemical Library for Research and Methods Development

- Attempted to procure ~3,000 based on chemical diversity, Agency priorities, and other considerations
- Obtained 480 total unique chemicals
  - 430/480 soluble in DMSO (90%)
  - 54/75 soluble in water (72%) (incl. only 3 DMSO insolubles)
  - Issues with sample stability and volatility
- Subset of PFAS Library for testing:

Hepatotoxicity
Developmental toxicity
Mitochondrial toxicity
Developmental neurotoxicity
Endocrine Disruption
General toxicity

Only a fraction of PFAS compounds disrupt network formation



- ~25% of tested compounds were active
- No PFAS compound increased network formation parameters compared to control wells
- Three Groups: **1**) “Pan Active” **2**) subset of parameters **3**) Inactive
- Positive and negative controls gave appropriate responses.
- Replicates gave generally consistent results
- Cytotoxicity was prominent in “Pan Active”

Figure courtesy of Kelly Carstens, EPA

Example #2: WoE for Glufosinate DNT Waiver

Problem Formulation

EPA's Office of Pesticide Programs (OPP) received notification that different parties intended to register L-glufosinate ammonium and L-glufosinate acid as pesticides (herbicides)

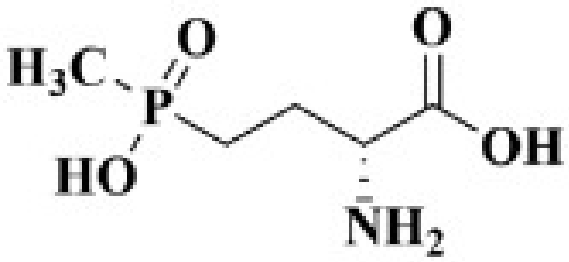
- DL-glufosinate ammonium was already registered as a pesticide, and a Guideline DNT study had been submitted to OPP
  - Decreased pup weight, **morphometry changes in hippocampus**, motor activity changes were reported
- DL-glufosinate also has acute neurotoxicity
- Literature report of **altered network activity following acute exposure** in vitro (Lantz et al., 2014)

Problem: Is the Guideline DNT for DL-glufosinate sufficient to inform decisions for L-glufosinate isomers?

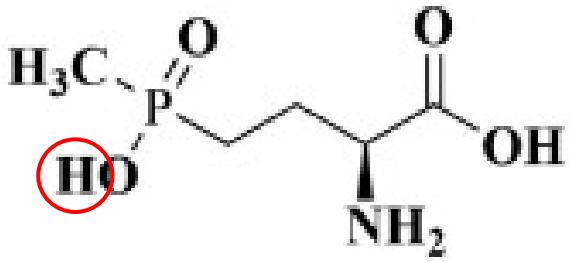
Need: Comparative bioactivity data for DL- vs L-Glufosinate isomers

Testing approach

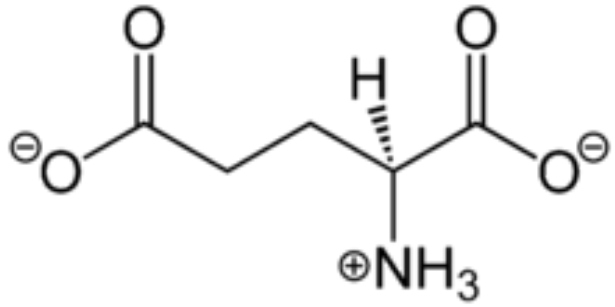
Compounds DL-glufosinate, L-glufosinate acid and L-glufosinate ammonium were tested, + assay controls



D-glufosinate



L-glufosinate



L-glutamate

Selected Assays for Glufosinate: Rationale

Neurite outgrowth in human iPS-derived neurons

Rationale-

Morphological changes observed in guideline DNT study

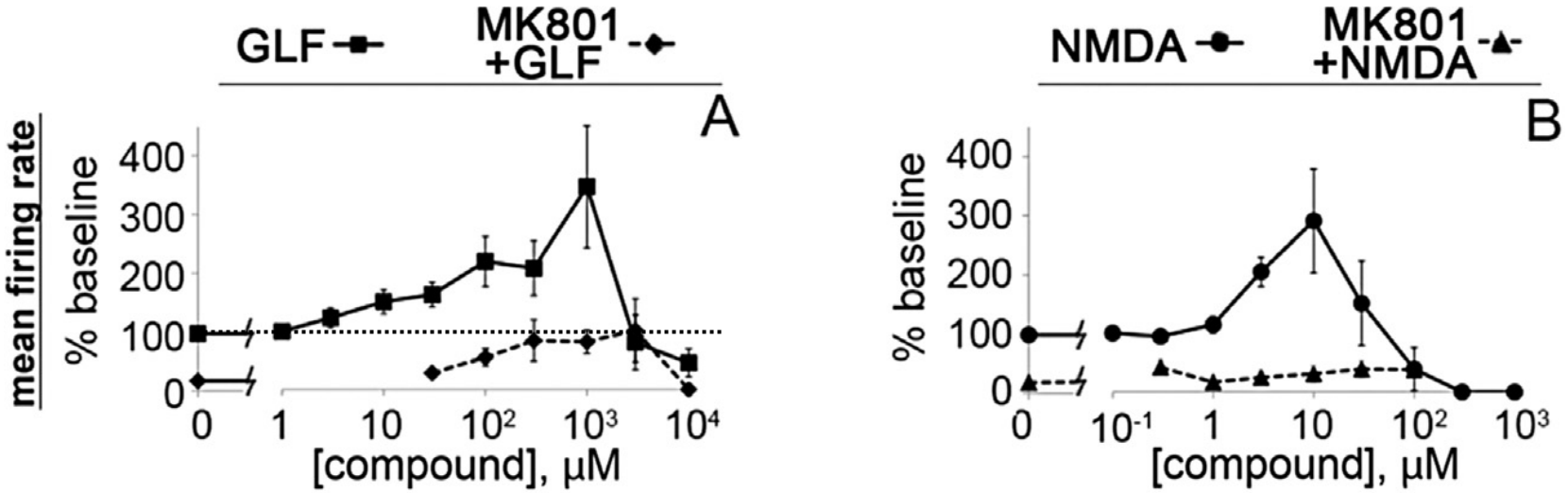
Ketamine, an NMDA antagonist, altered NOG in a human cell model

Network Formation Assay

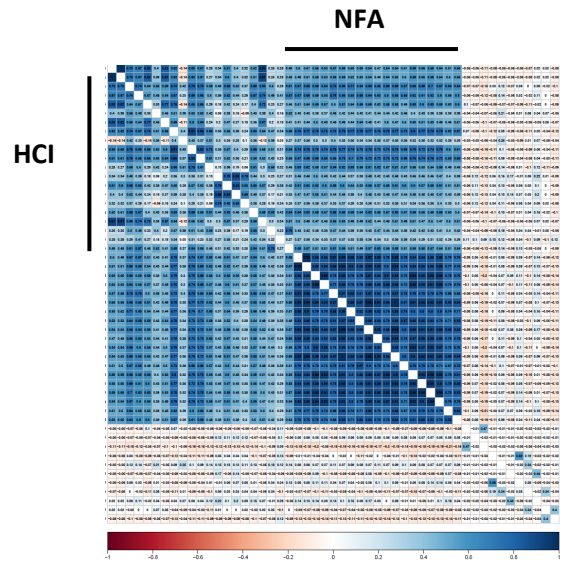
Rationale-

Effects of glufosinate on network function via NMDA Receptors following acute exposure in vitro

High correlation between outcomes in NFA and other HCI assays (Proliferation, NOG, Synaptogenesis)

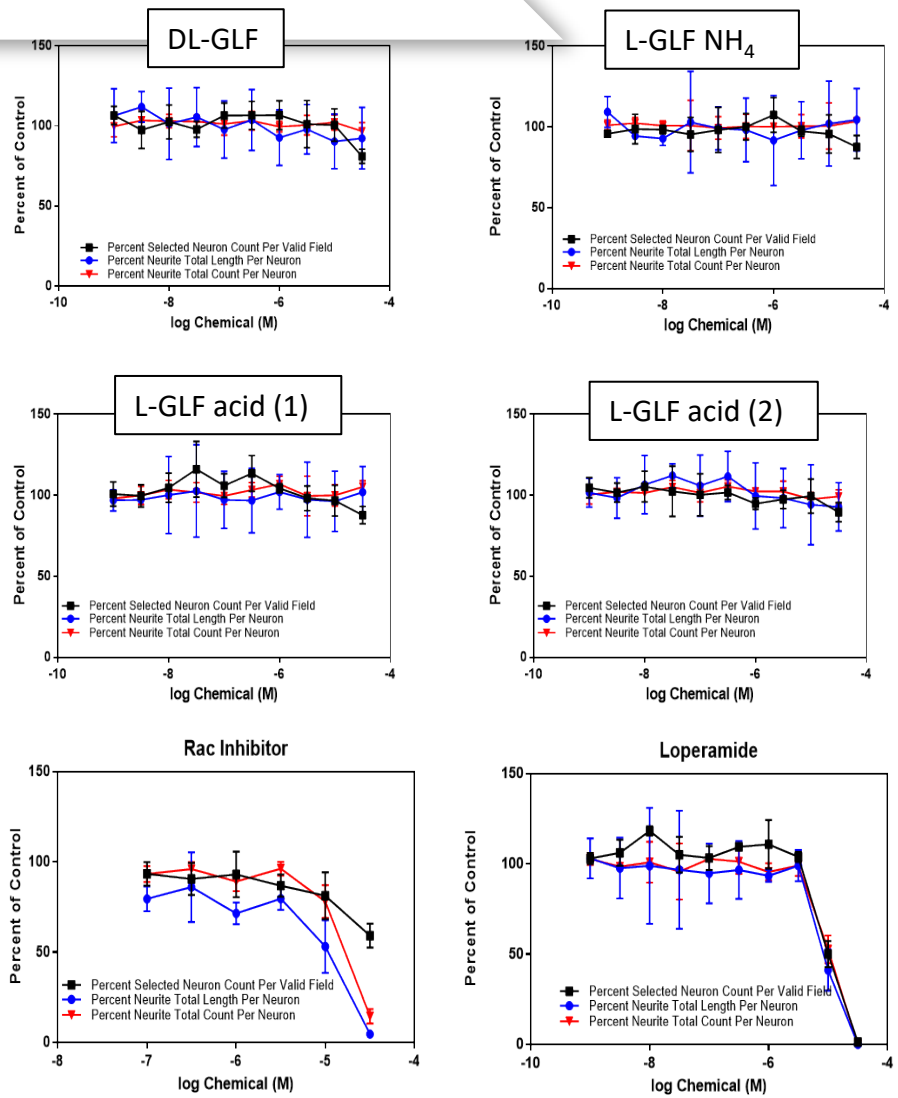


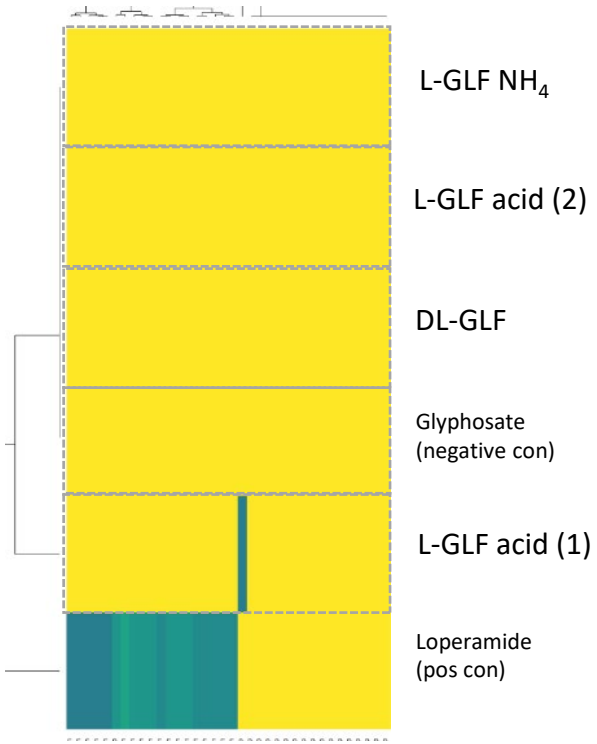
Lantz et al., 2014

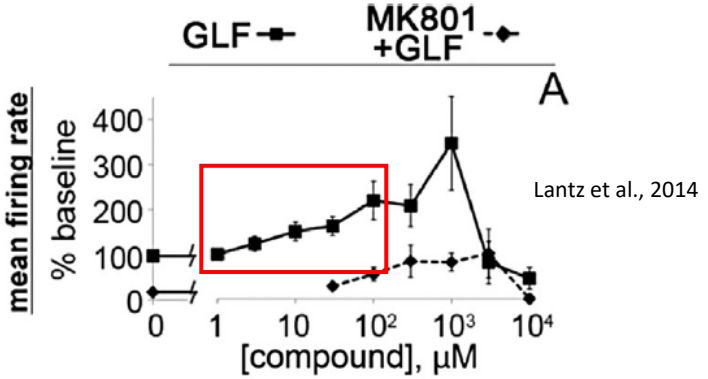


Carstens et al, Toxicol Sci 2022.

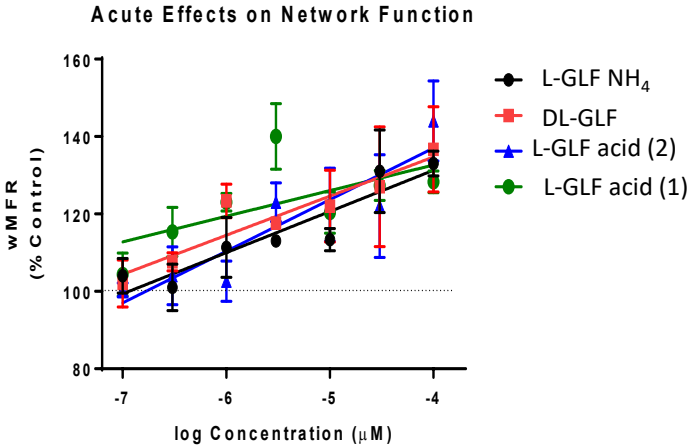
Using WoE and DNT NAMs for Guideline DNT waiver decisions







Lantz et al., 2014



From Guideline study, NOAEL of DL-GLF = **14 mg/kg/day**

Using HTTK and IVIVE

- 1 mg/kg/day = Css values of 0.66 and 2.21 µM in rats and humans, respectively
- 30 µM DL-GLF = AED of **45 mg/kg/day** (rats) and 13.5 mg/kg/day (humans)

Dobreniecki et al 2022. Reg Toxicol Pharmacol 131

Using WoE and DNT NAMs for Guideline DNT waiver decisions

In vitro evidence

- Lack of effect on neurite outgrowth in human cells
- Lack of effect on network formation in rat cortical networks
- Positive effects on acute network activity demonstrate biological activity and add confidence to the lack of effects in DNT-related assays (neurite outgrowth and network formation)
- Similar effects of DL- and L-isoforms in all in vitro assays

In vitro to in vivo extrapolation (IVIVE)

- Tested concentrations in vitro > PODs selected for L-glufosinate risk assessment

In vivo evidence

- Existing guideline DNT study for DL-glufosinate showing effects on morphometry, motor activity and pup weight
- Non-guideline DNT for L-glufosinate showing increased motor activity, decreased body wt in pups (morphometrics not conducted)
- Comparable toxicity profiles for both DL- and L-glufosinate.

Dobreniecki et al 2022. Reg Toxicol Pharmacol 131

Using WoE and DNT NAMs for Guideline DNT waiver decisions

Risk Calculations

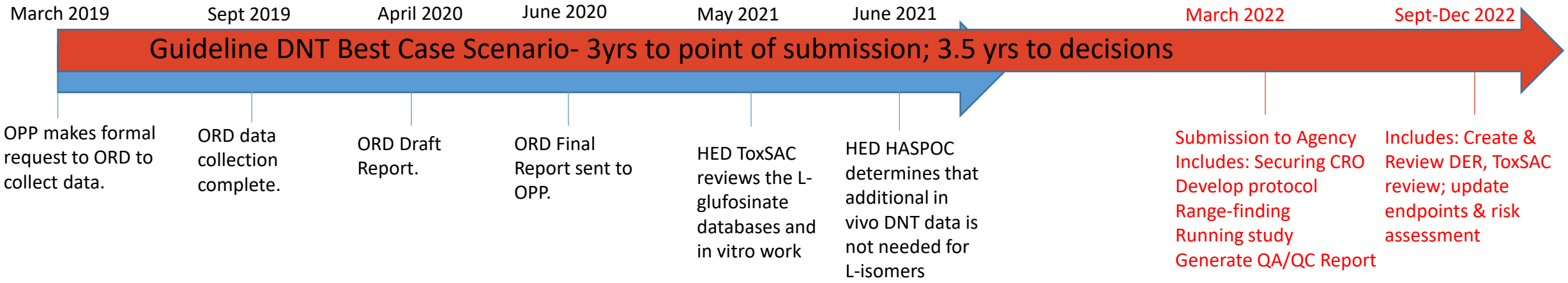
- Point of Departure (POD) was 30x lower than calculated AED from in vitro studies (which were without effect)
- %Population adjusted doses (%PAD) < 100% (for dietary exposures)
- Margin of exposure (MOE) > Level of concern (LOC) for non-dietary exposures

CONCLUSION: Additional in vivo data would not likely identify a lower POD or more sensitive endpoint for isomer risk assessments

DECISION: Waivers granted for guideline DNT studies for L-glufosinate acid and L-glufosinate ammonium

Dobreniecki et al 2022. Reg Toxicol Pharmacol 131

Comparison to a DNT Guideline study- Impacts of the Decision



Animals Used:

- In vitro study- 3 Pregnant Dams (~12-15pups)
- Guideline study- 160 Pregnant Dams (2 compounds X 3 doses + control @20/dose (recommended))
  - ~1600 pups

Cost:

- In vitro study- $1000 for Assays + $96,000 labor = $97,000
- Guideline study- $2,000,000 (2 compounds x $1M each)

Conclusions

The development of a DNT-NAM battery for assessing potential DNT hazard:

- Provides an opportunity to overcome some of the challenges with the *in vivo* DNT guideline
- Evaluates critical processes underlying neurodevelopment
- Incorporates human relevant information

DNT NAMs are being utilized at the EPA for a variety of regulatory decision-making processes

Thank you!
Questions?

EPA ORD Colleagues:

- Kathleen Wallace
- Theresa Freudenrich
- Bill Mundy (retired)
- Josh Harrill
- Jasmine Brown
- Katie Paul Friedman
- Melissa Martin
- Kelly Carstens
- Amy Carpenter (ORISE)
- Seline Choo (ORISE)
- Richard Judson
- Grace Patlewicz

EPA Program Office Colleagues

- Anna Lowit
- Liz Mendez
- Monique Perron
- Sarah Dobreniecki
- Mike Metzger