Applying assays from the In Vitro Developmental Neurotoxicity Testing Battery to Compare DL- to L-Glufosinate Activity

Timothy J Shafer, PhD

Biomolecular and Computational Toxicology Division
Center for Computational Toxicology and Exposure

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Phone: 919-541-0647
Shafer.tim@epa.gov

This Presentation Does not Represent Agency Policy

I.   Background on the history and philosophy of the developmental neurotoxicity in vitro assay battery (DNT_IVB)
II. Neurite Outgrowth (NOG) Assay in human IPS-derived neurons
III. Network Formation Assay (NFA) in rat cortical cells grown on microelectrode arrays (MEAs)
IV. Example Data from the DNT_IVB
V.   Rationale for evaluating DL-Glufosinate (DL-GLF) and purified isomers in NOG and NFA.

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New Approach Methodologies (NAMs) are Needed for Developmental Neurotoxicity (DNT) Hazard Characterization

Guideline DNT studies are resource intensive, time-consuming and often do not produce actionable data. DNT hazard evaluation has lagged for numerous chemicals due to the high cost and low throughput of this approach.

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

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

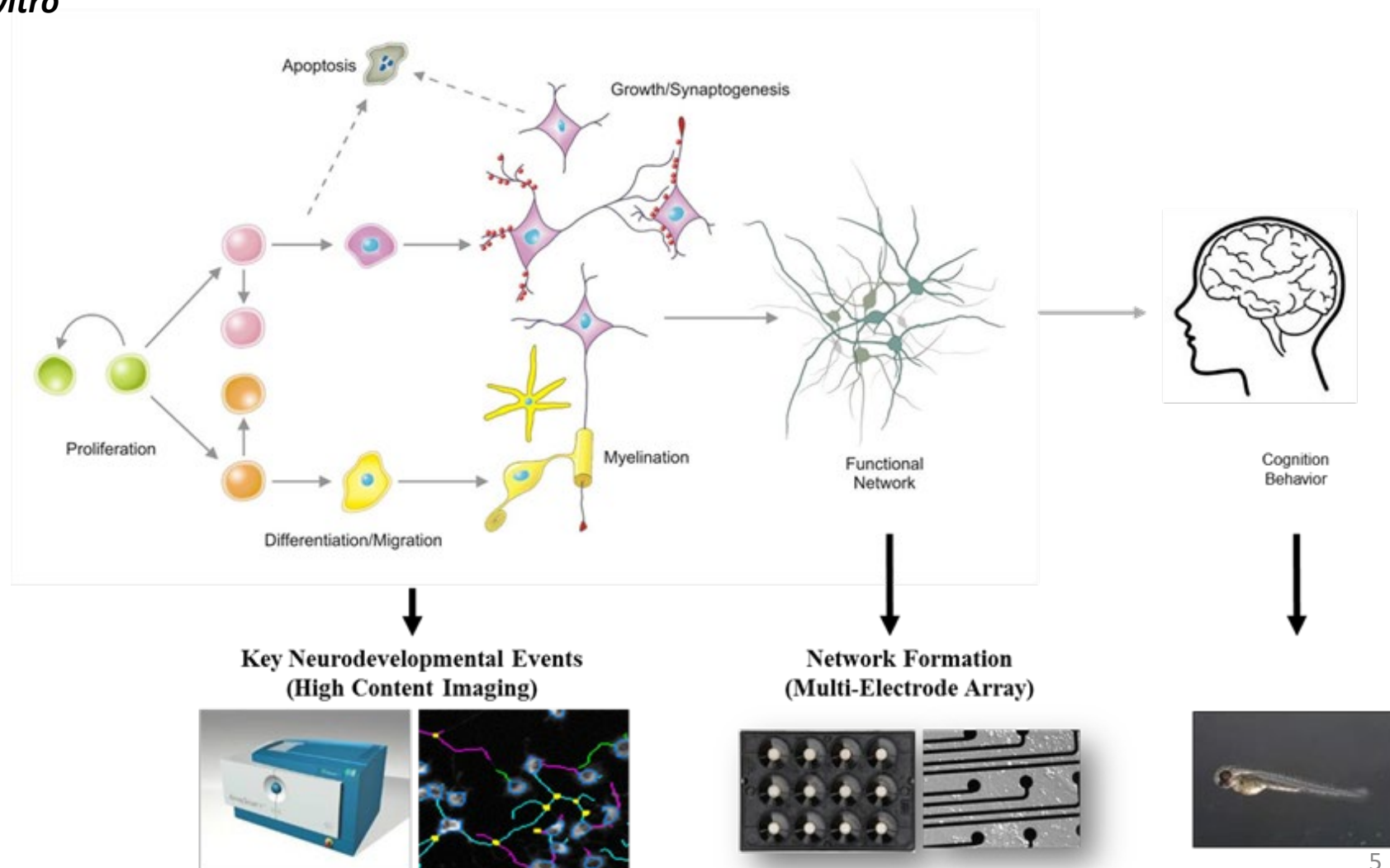- Data from these assays can provide information for decision-making

- 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*

Hypothesis: *If a compound alters a neurodevelopmental process in vitro, then there is a concern it could be an in vivo DNT hazard*
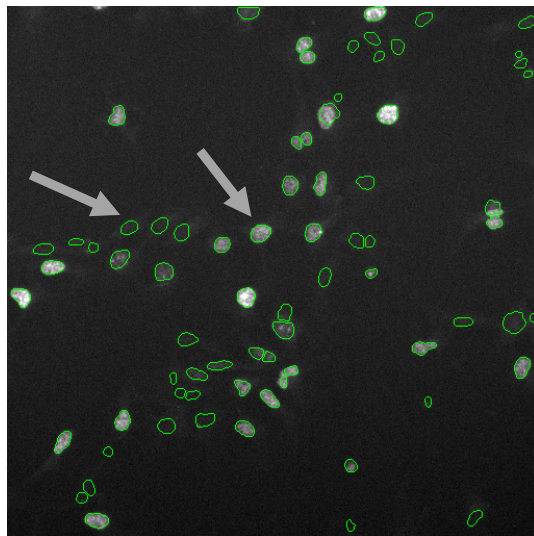
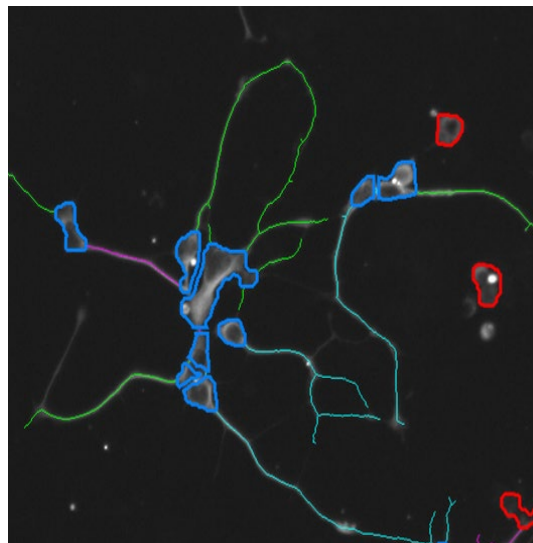**Quantify key neurodevelopmental events *in vitro***

Criteria for assays

- Evaluate key neurodevelopmental processes *in vitro*

- Develop assay method using cell-based endpoint that is amenable to high throughput/content testing.
  - -use "assay positive control" compounds.

- Evaluate ability of assay to detect changes in key events using a "test set" (chemicals with known effects on DNT *in vivo*)

- Assessment of cell health/viability (cytotoxicity assays).

- Test Environmental Chemicals over a broad concentration range (10^{-9} to 10^{-4} M).

Apoptosis

Growth/Synaptogenesis

Proliferation

Differentiation/Migration

Myelination

Functional Network

Cognition Behavior

Key Neurodevelopmental Events (High Content Imaging)

Network Formation (Multi-Electrode Array)

Proliferation

Neurite Outgrowth

Synaptogenesis
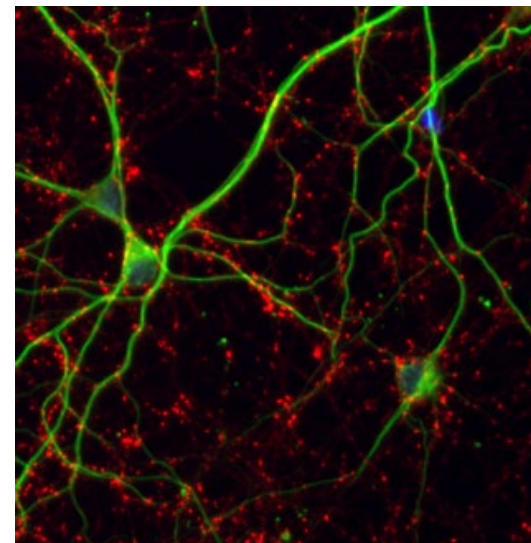
Apoptosis
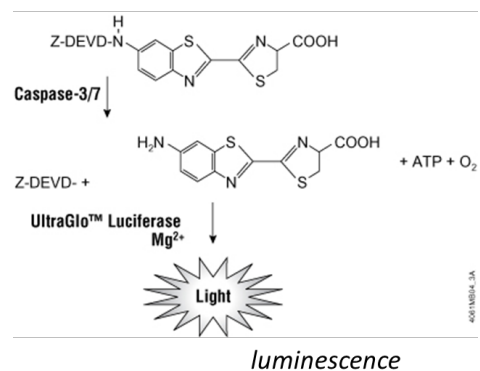
Network Function and Formation

luminescence

Apoptosis
Apop

Synaptogenesis
Synap

Neurite growth
UKN4 & 5
RatCort_NOG
iCell_NOG

MEA-NFA
MEA-AcN

Differentiation
UKN2
NPC3-5

Myelination
NPC6

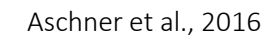Proliferation
hNP1

UKN2
NPC2   Migration

Neural network
formation & function

Aschner et al., 2016
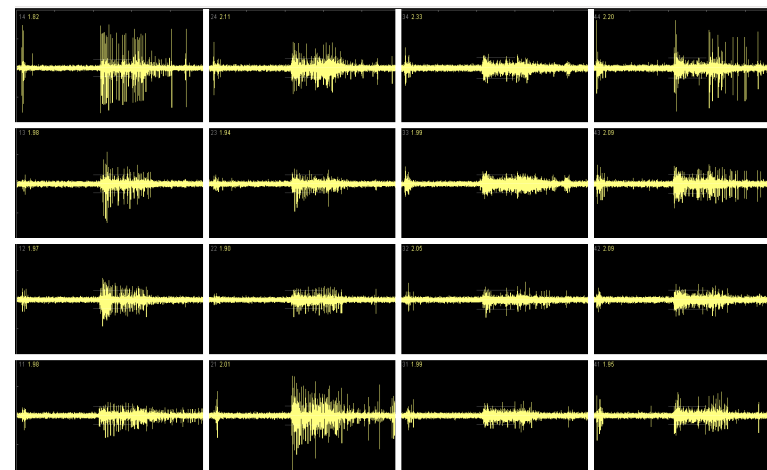
II & III. Introduction to the iCell Neurite Outgrowth (NOG) and rat cortical Network Formation Assay (NFA)

High Content Imaging of Neurite Outgrowth

- Culture cells in 96 well plate and expose to chemicals for 48hr.
- Fix and ICC to detect nuclei, cell body, neurites using fluorescent imaging

Channel 1 (Hoechst)　　　　Channel 2 (β-tubulin)　　　　Analysis
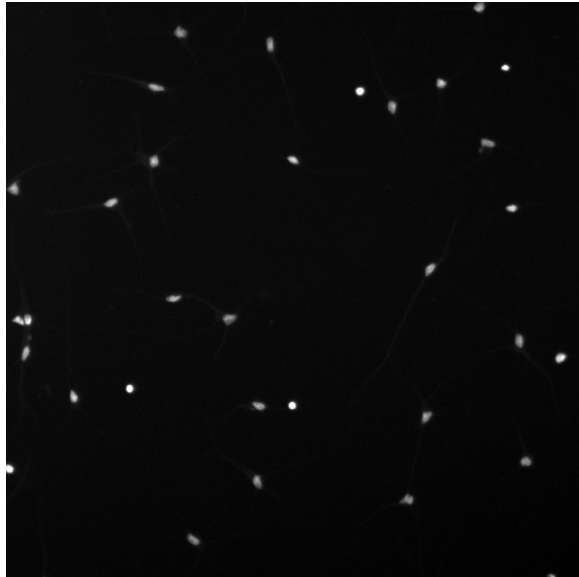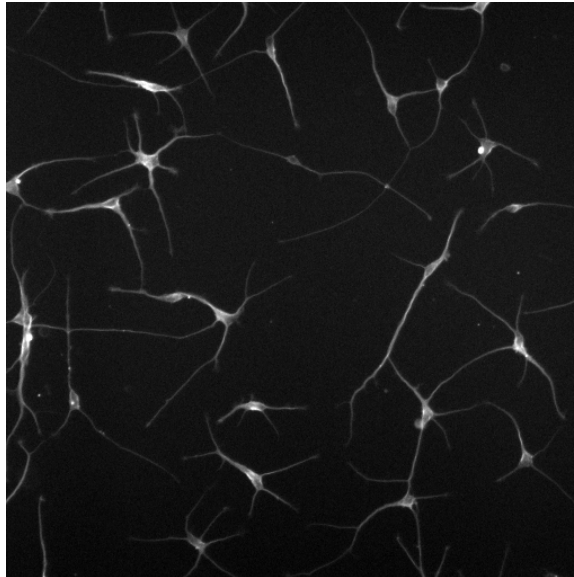
nuclei　　　　cell bodies and neurites　　　　mask

(Data for 300 cells/well in 96 well plate in 30 min of imaging time)
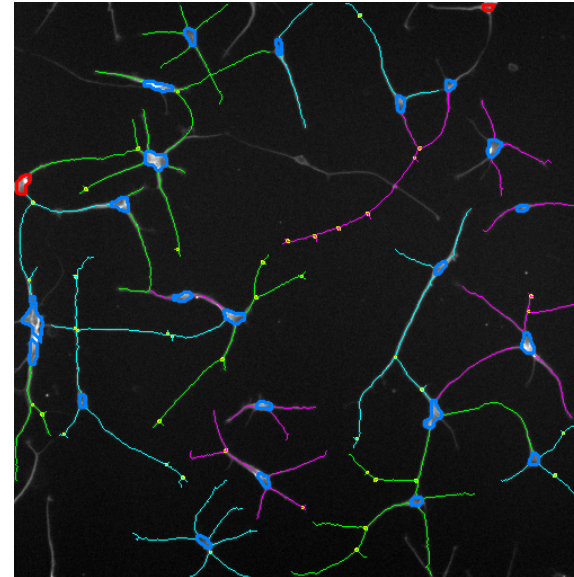
Endpoints:
- Cell Number
- #Neurites/cell
- Neurite length
- Neurite branching

I_{gluta} Neurons Are Polarized and Include Inhibitory Neurons

β-tubulin/pNeurofil. (axon)
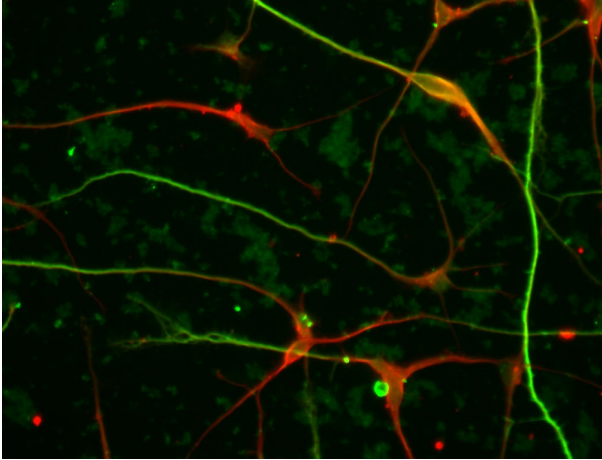
MAP2/GABA

- Pan-axonal antibody identifies putative axons
- ~75/25% Glutamatergic/GABAergic
- Cells exhibit rapid neurite outgrowth
- Performance metrics also indicate that assay can detect chemical-induced changes in neurite outgrowth.
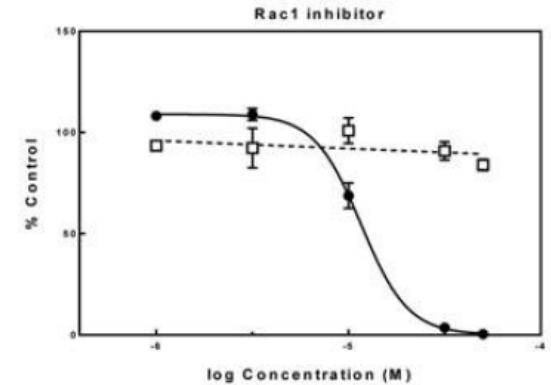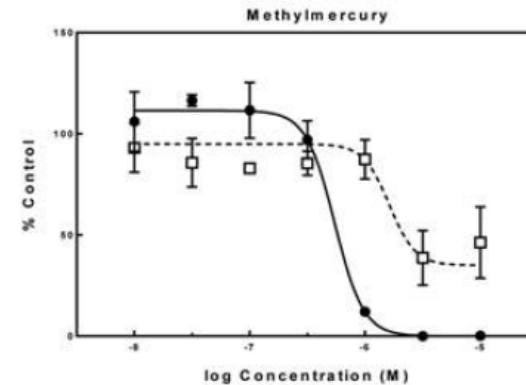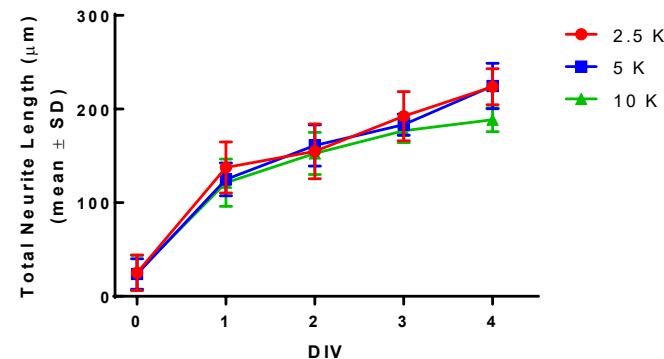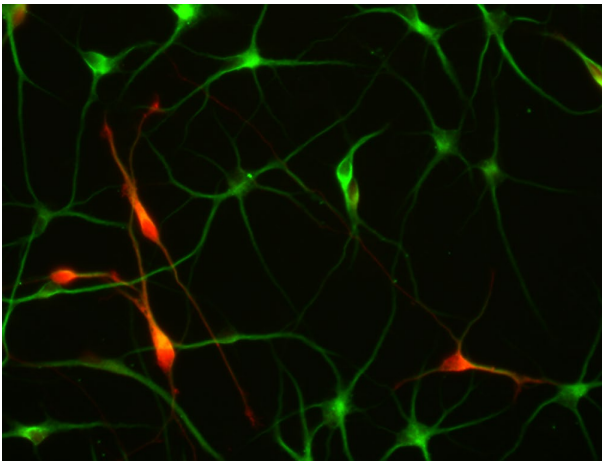
● Total Neurite Length/neuron　　□ Neurons/Field

From Druwe et al., 2016

"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

50 μm

200 μm

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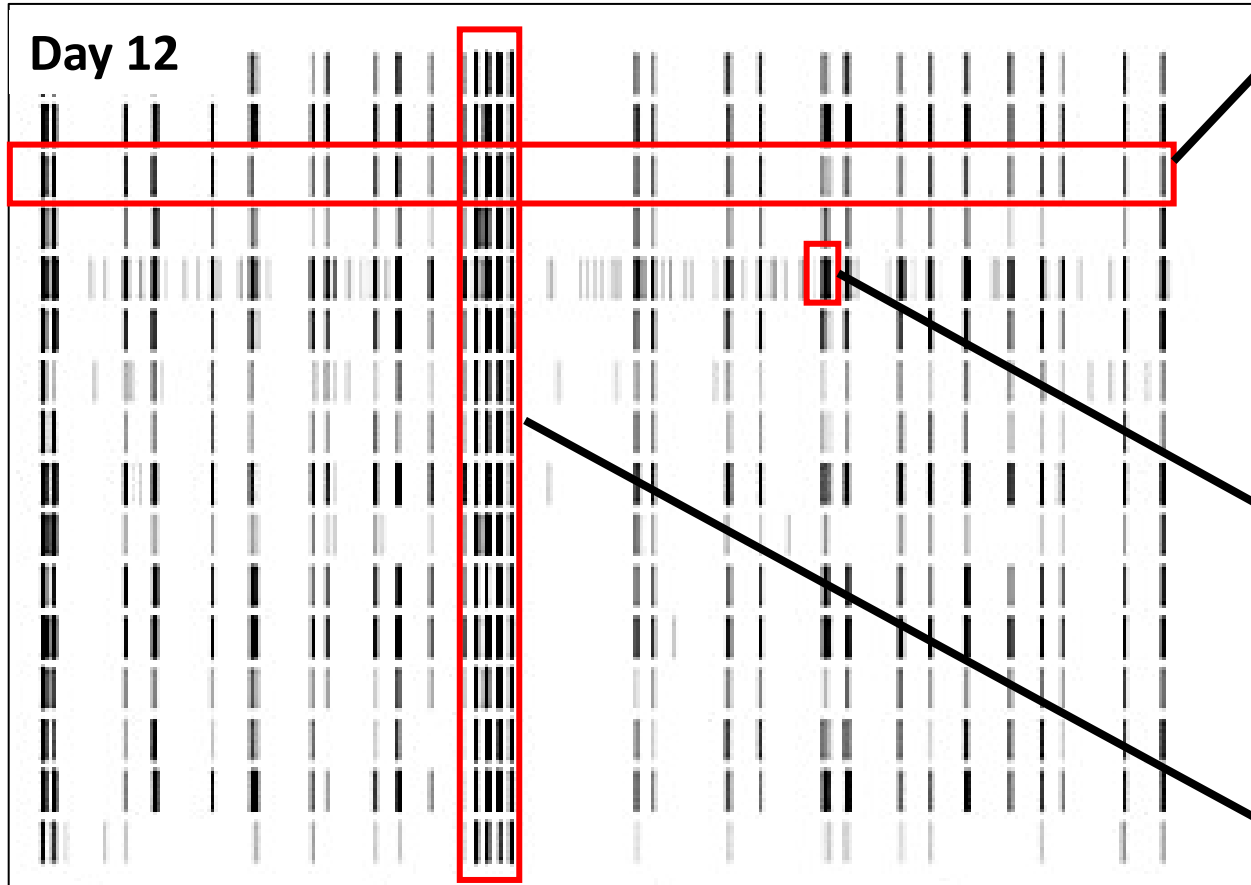- Repeated recordings from same sample

The electrical activity recorded by MEAs are the biological underpinnings of EEG recordings.
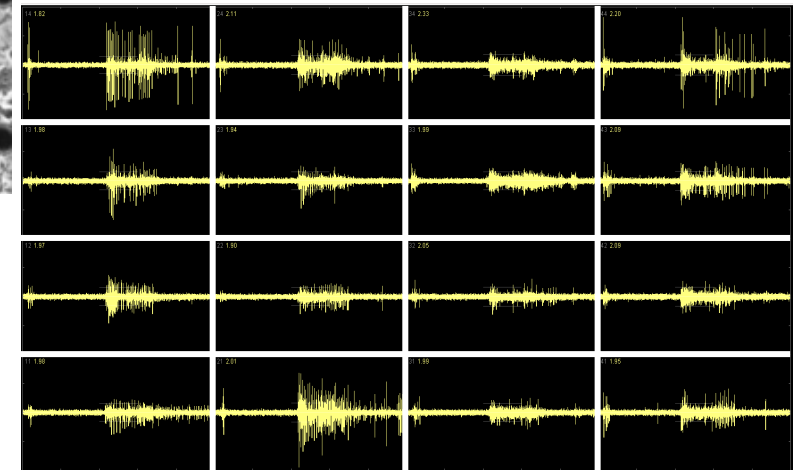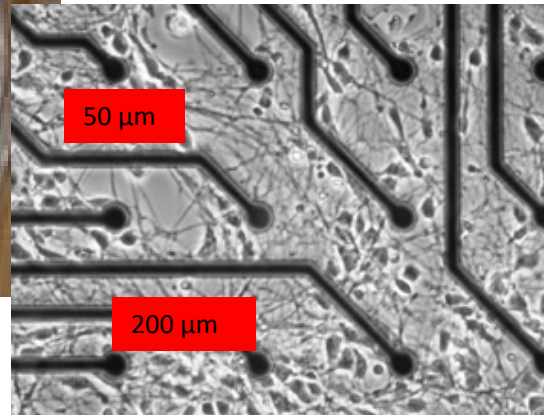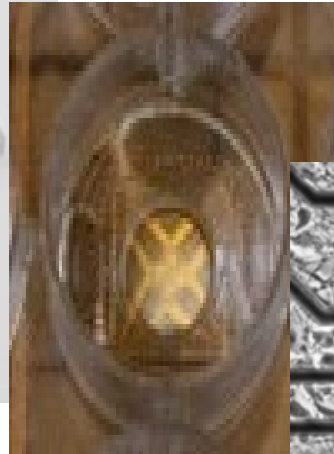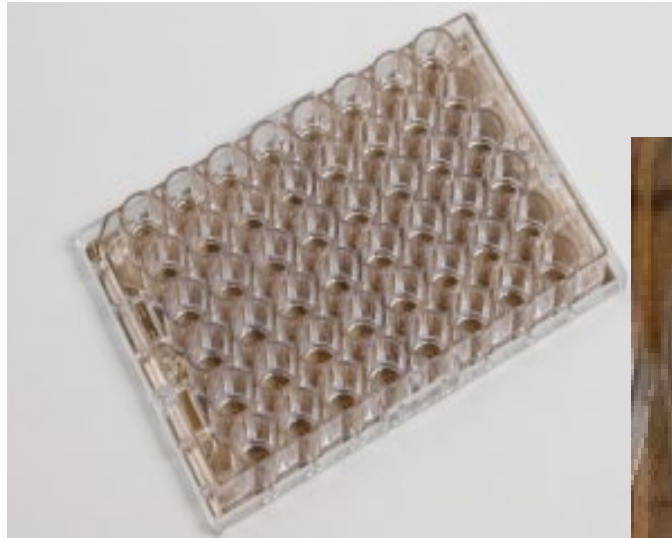
A snapshot in time of neural network activity in one well. Each box represents the electrical activity of neurons on 1 electrode in the array.

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.
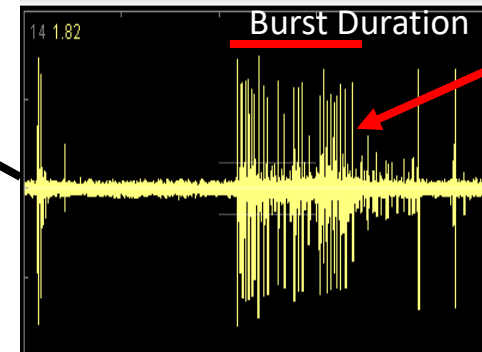
Number of Action Potential "Spikes"/burst

Burst Duration

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

Day 12

- Spiking, bursting, and synchronous activity are intrinsic network functions.
  - These properties of networks develop spontaneously *in vivo* and *in vitro*

- Neuro-developmental processes are influenced by electrical activity.

- Patterns of network activity are highly conserved.
  - There is greater similarity across the same region of brain from different species than between brain regions of the same species

- Synchronous activity in networks is integral to sensory awareness, attention, memory and other cognitive processes.

Primary Cultures of Cortical Neurons are Complex and Representative of in vivo Cortex

*excitatory (red) : inhibitory (green) neurons 65%:35%*
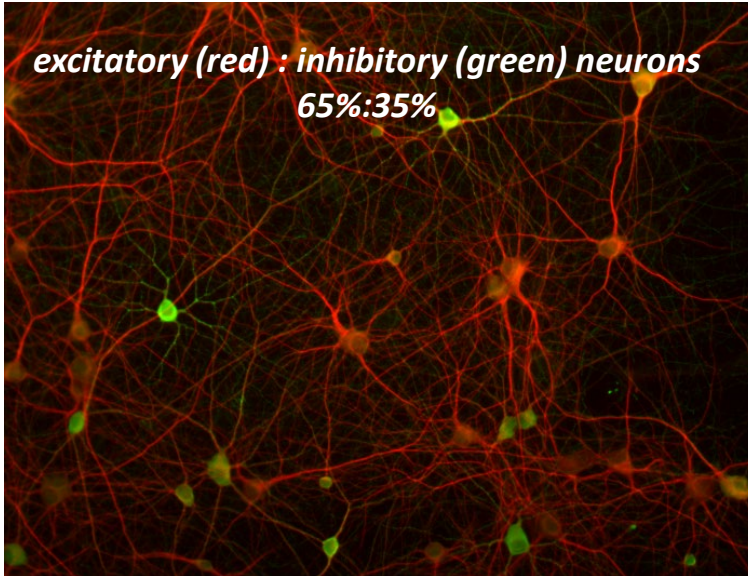
- Glutamatergic and GABAergic neurons, glia, oligodendrocytes.
- Shift in ontogeny of excitatory/inhibitory responses recapitulates cortical development in vivo.
- Responsive to NMDA, AMPA, Kainic acid, GABA, Dopamine, Voltage-gated Na and Ca ion channels.
- Ontogeny of network activity between DIV 0 and 12.
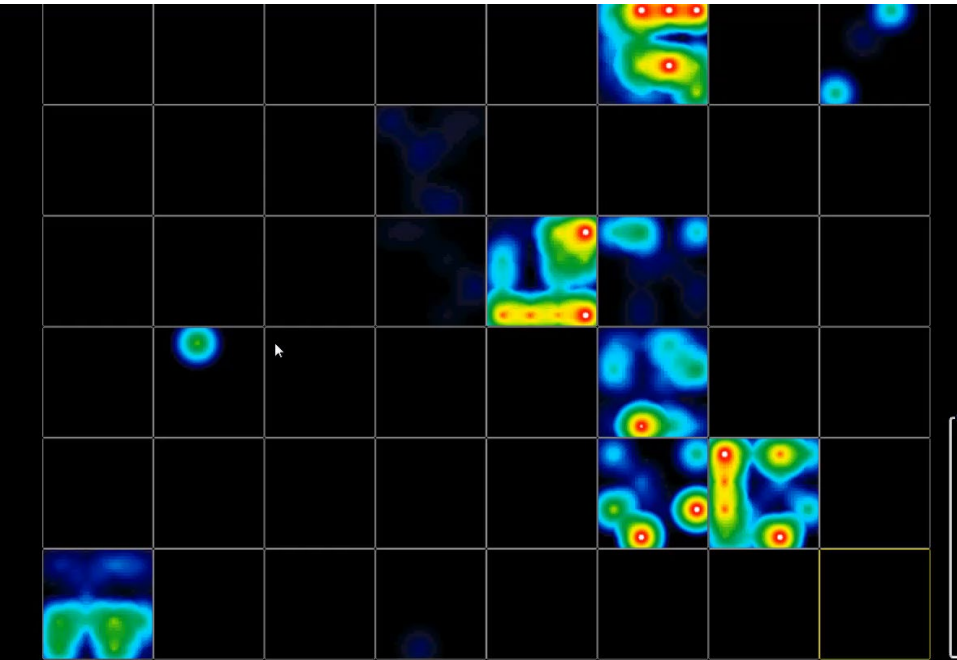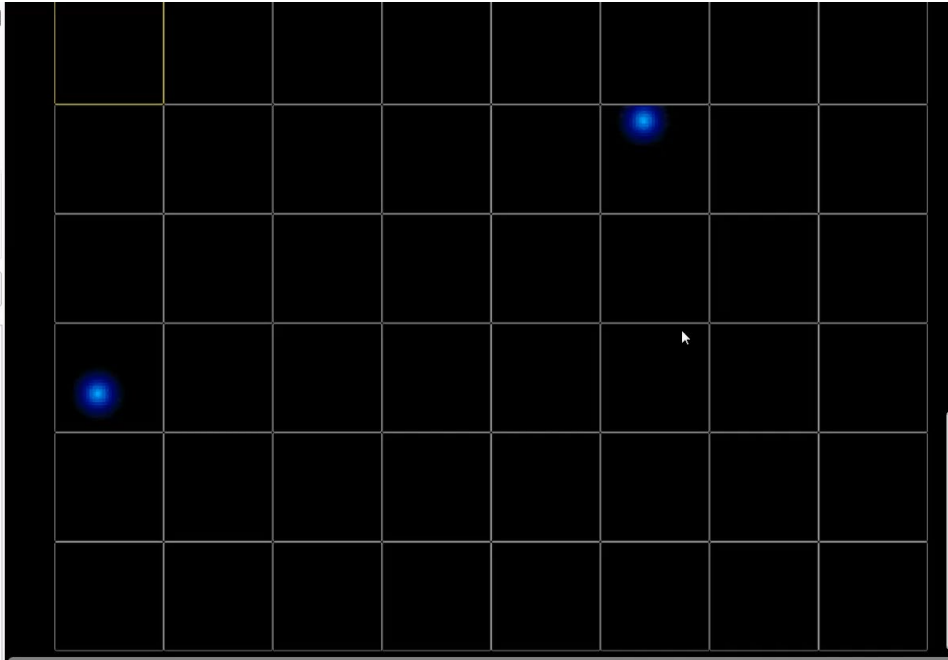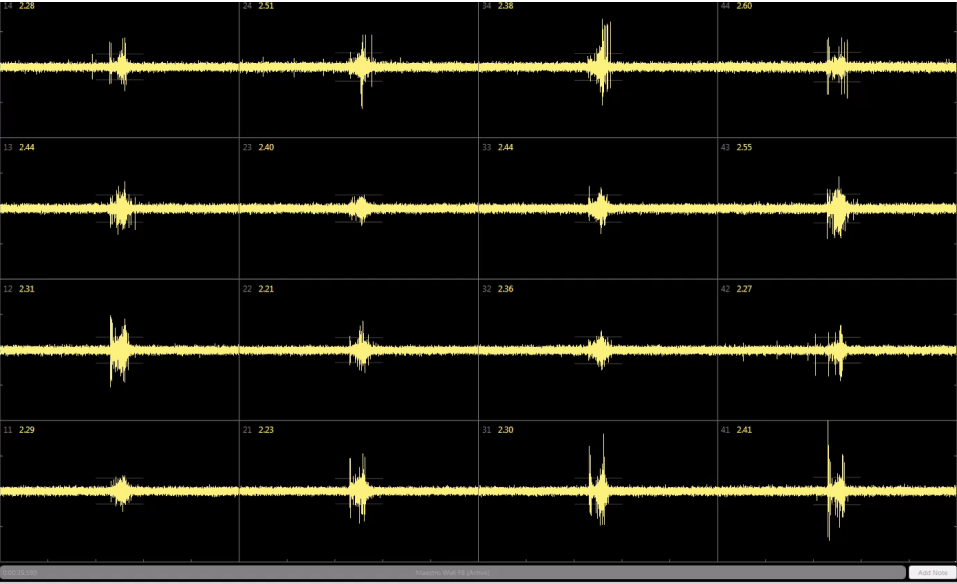- Good Performance Metrics (control variability, Z' scores, replication of duplicate samples)

Inglefield and Shafer, 2000
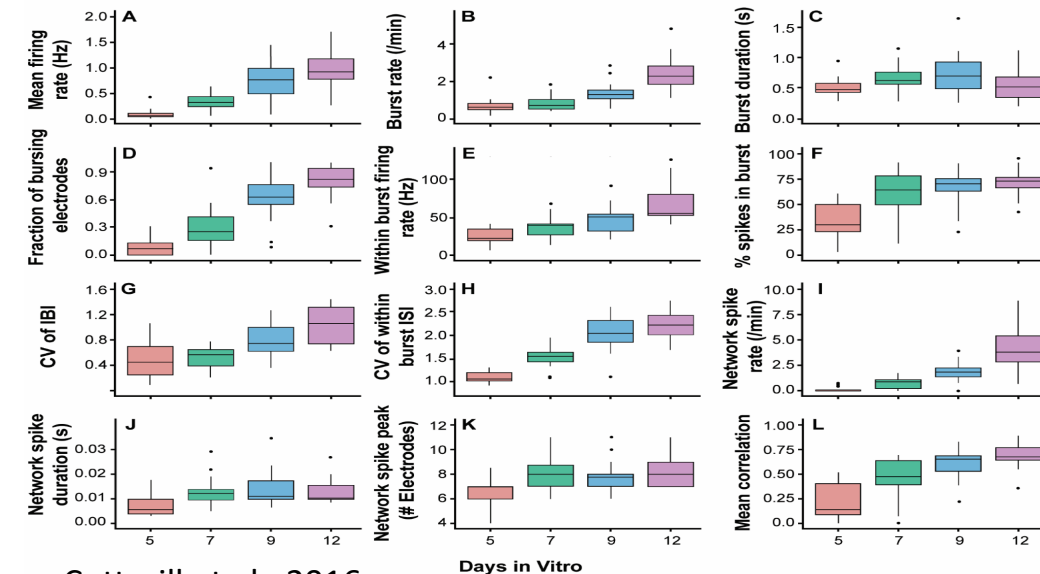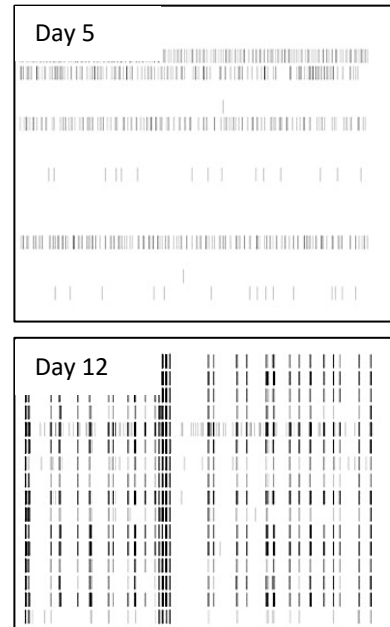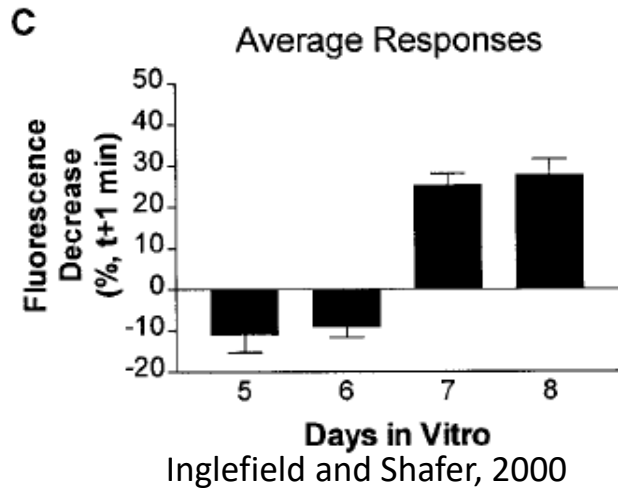
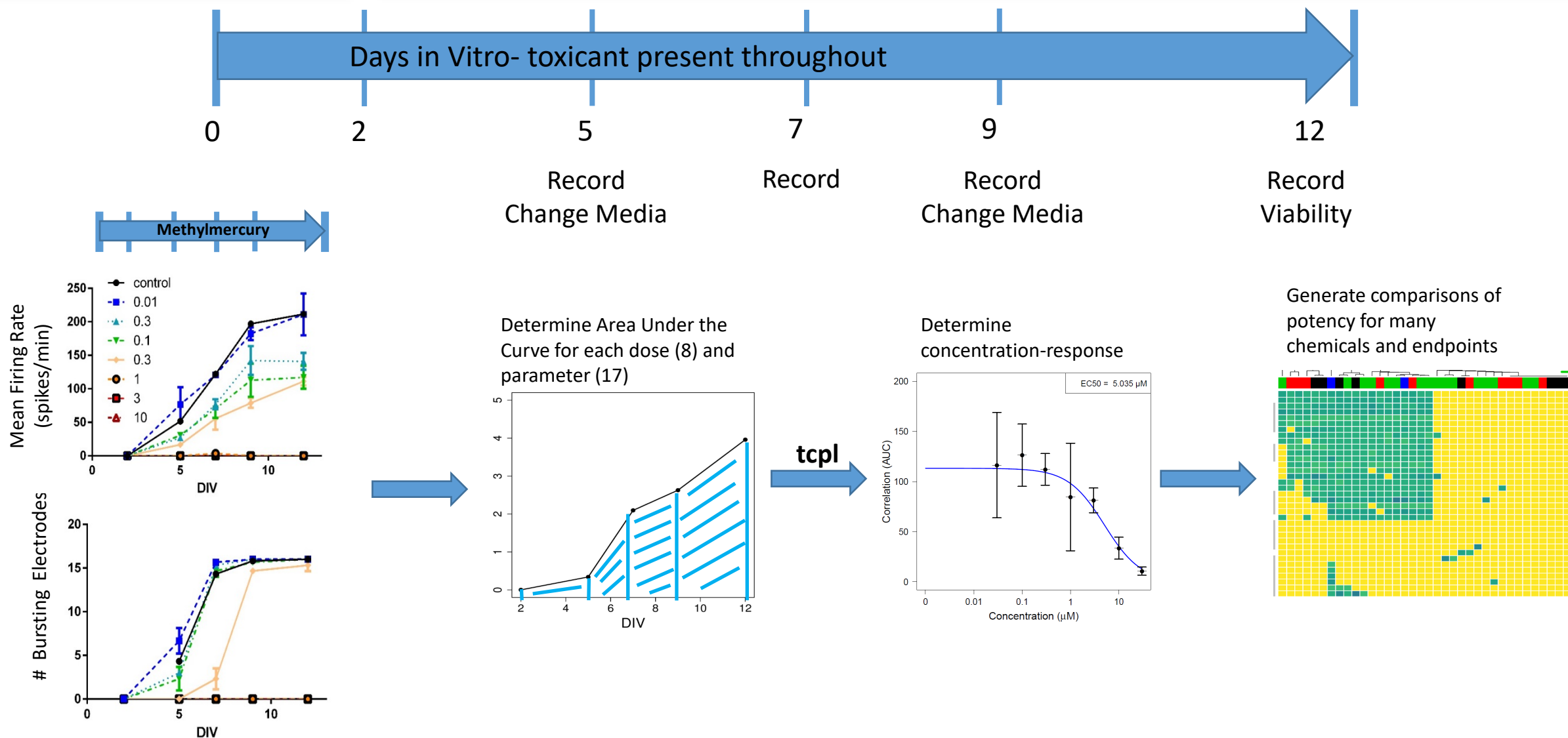Cotterill et al., 2016

5-Days old
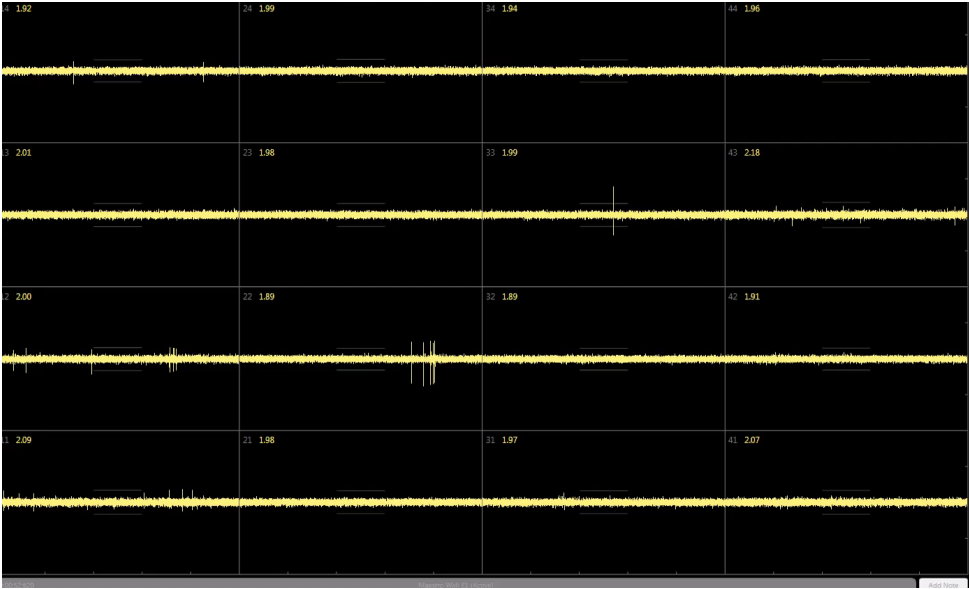
12-Days old

Testing Chemicals for Effects on Neural Network Formation: The Assay Protocol in Brief

IV. Example Data from the Developmental Neurotoxicity in vitro Battery (DNT_IVB)

Sensitivity/Specificity Analysis for EPA DNT NAMs
55 DNT Reference and 13 DNT Negative Compounds

Activity Type
- NOG initiation, rat
- Synaptogenesis/maturatic
- NOG initiation, hN2
- Proliferation, hNP1
- General
- Bursting
- Network Connectivity

	Strong selectivity	Moderate selectivity	DNT Reference	
			Negative	**Positive**
1	Decreased network and general activity	Bursting, proliferation	1	12
2	Synaptogenesis, NOG (hN2)	NOG (rat), proliferation, decreased network and general activity	0	14
3		Moderate to low activity across endpoints	0	10
4		Inactive/ equivocal	12	17

		Negatives	**Positives**
Results from DNT-NAM battery	**Selective activity** (Clusters 1,2,3)	False positive:1	True positive: 36
	Inactive/ equivocal (Cluster 4)	True Negative: 12	False negative: 17

Sensitivity= 68%, Specificity= 92%, Accuracy= 73%
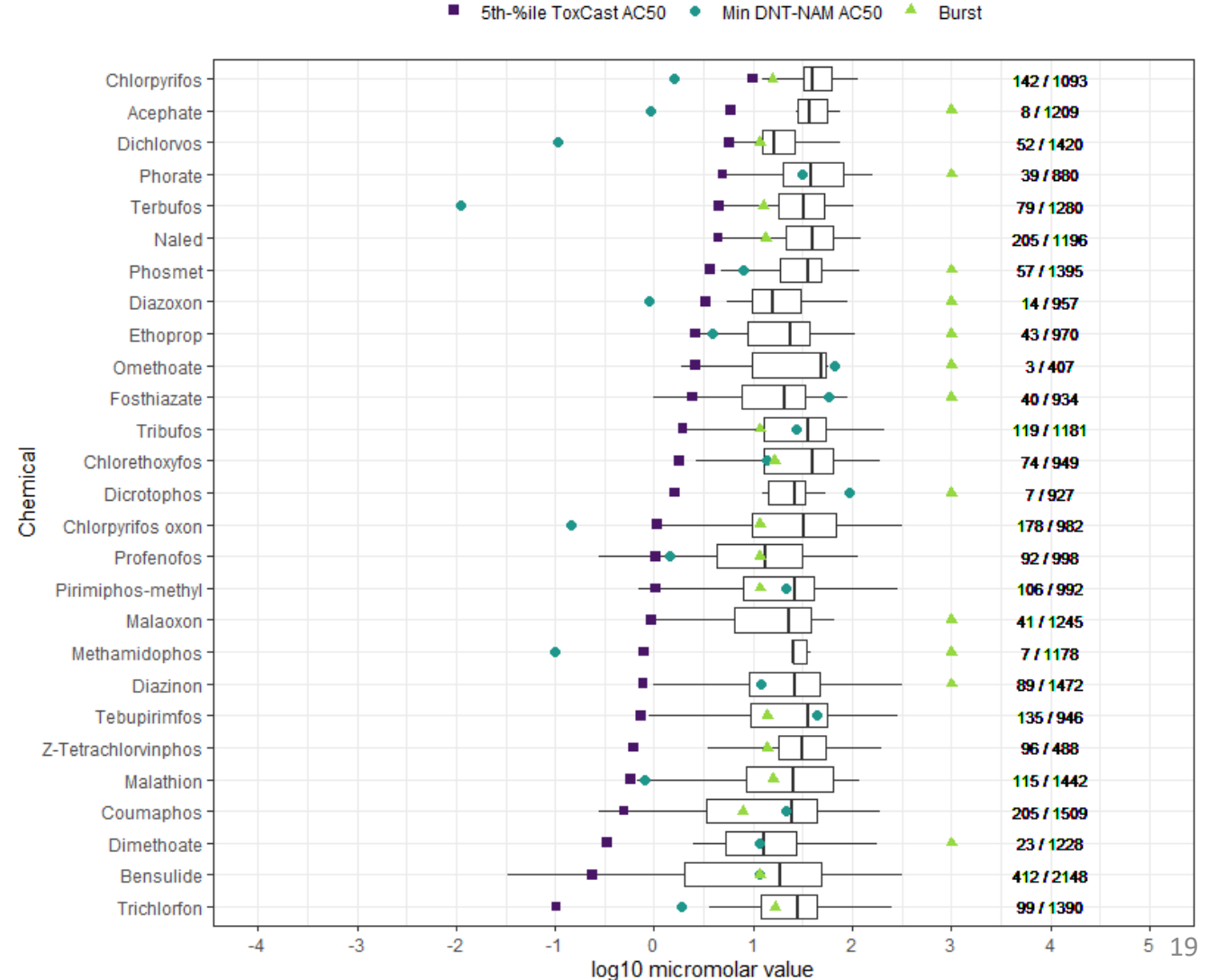
Carstens et al., in preparation

For some OPs, DNT-IVB AC_{50} < bioactivity estimate from the rest of ToxCast.

DNT-IVB may provide a more potent estimate of bioactivity for substances with minimum DNT-NAM AC50 < 5th percentile of filtered ToxCast AC50 values:

- Chlorpyrifos and chlorpyrifos oxon
- Acephate
- Dichlorvos
- Terbufos
- Diazoxon
- Methamidophos

Suggests that the DNT-IVB, in covering some new biology not previously in ToxCast, may yield bioactivity threshold concentrations lower than what is already available for some neuroactive substances in ToxCast.

These data indicate that DNT_IVB can be sensitive indicators of potential disruption of nervous system development
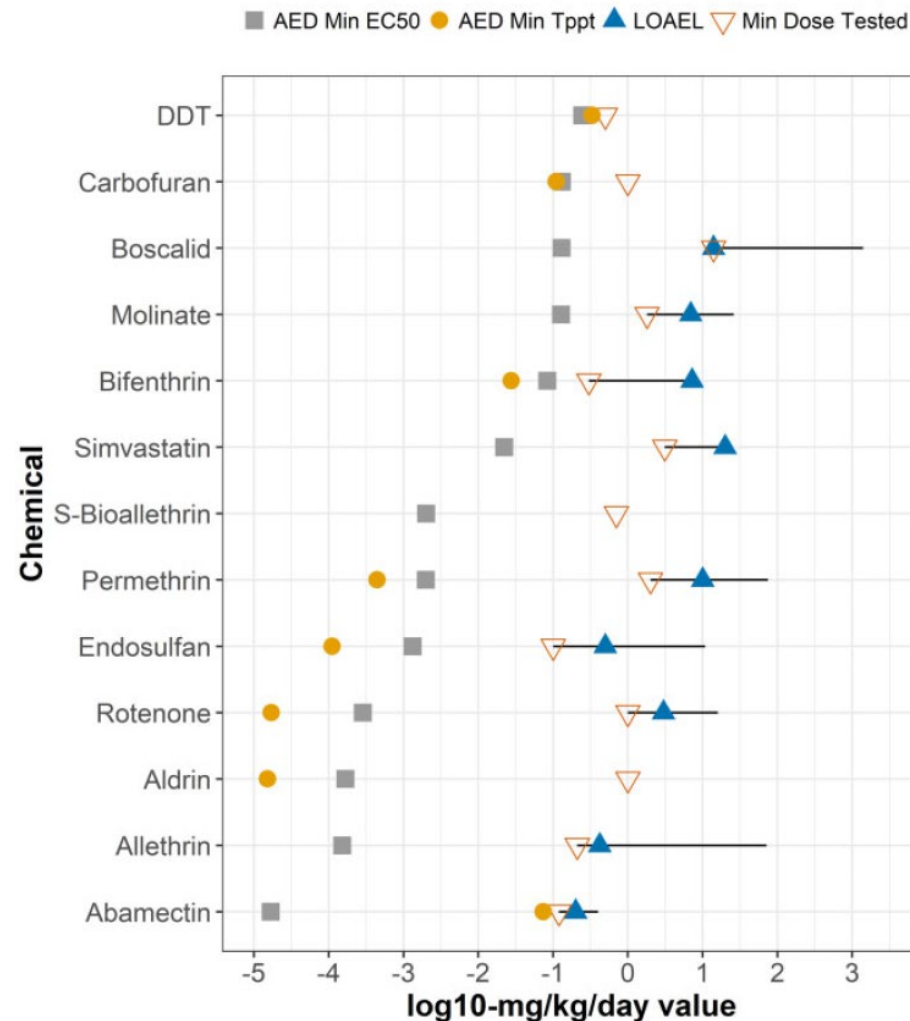
Neurite outgrowth in human iPS-derived neurons

Rationale-

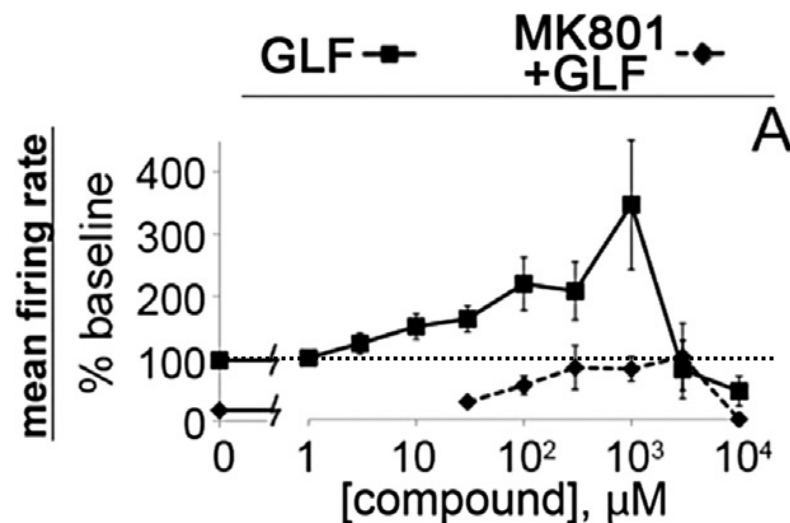Morphological changes observed in guideline DNT study

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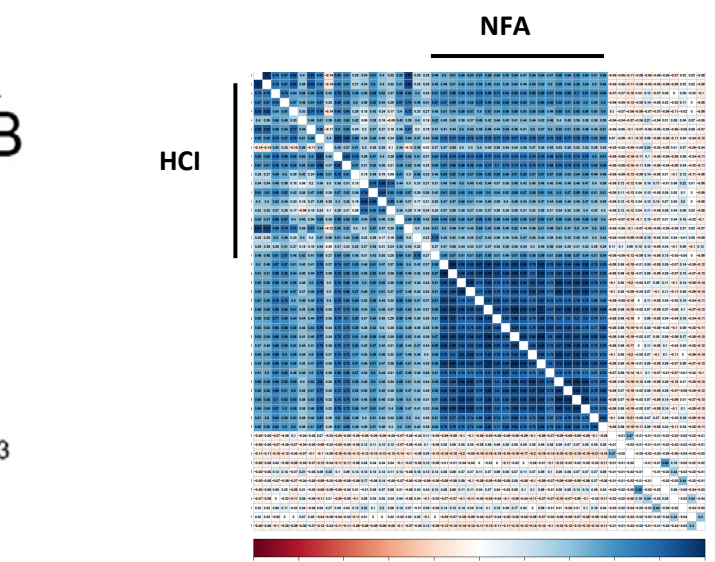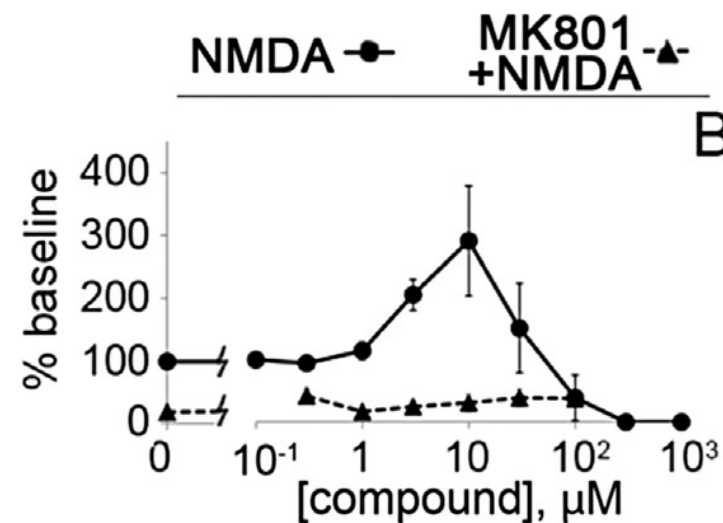
Network Formation Assay

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)
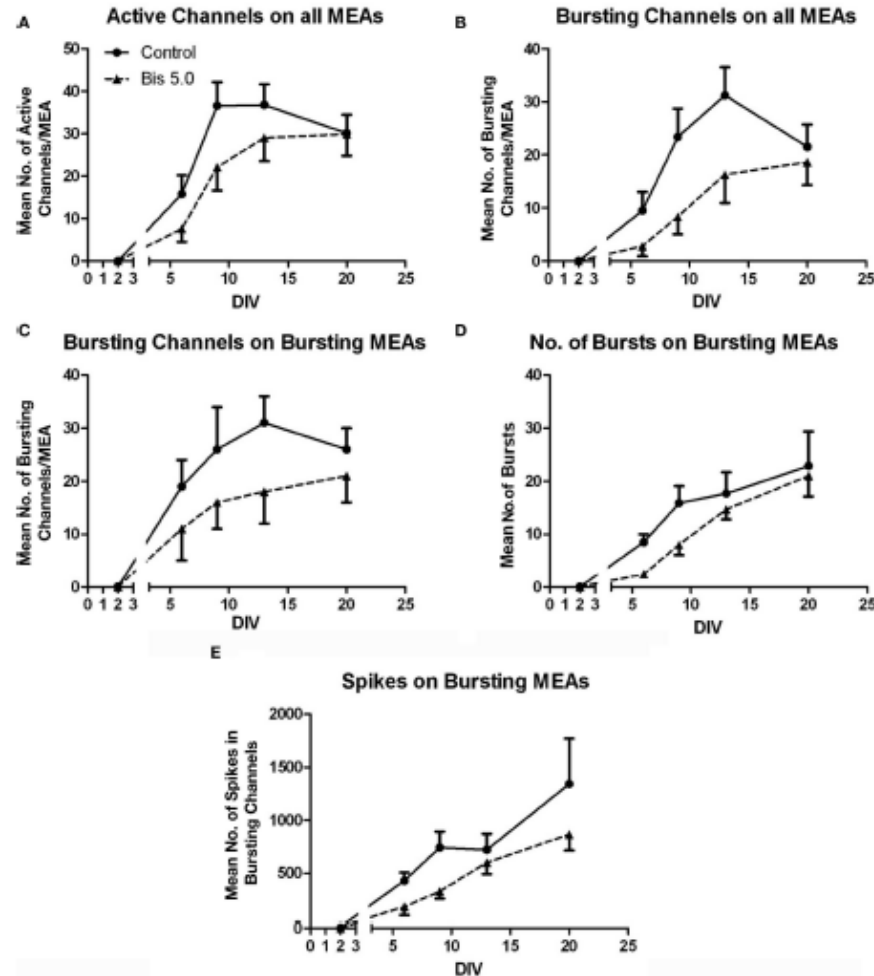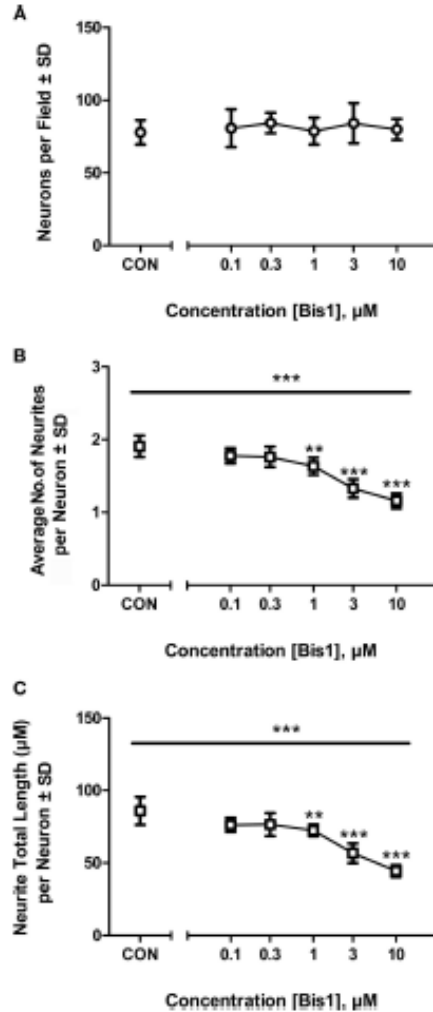

Lantz et al., 2014

Carstens et al, in prep.

Bisindolylmaleamide (Bis1) treatment of primary cortical cultures for 48 hr:
- Is NOT cytotoxic
- Decreases neurite outgrowth

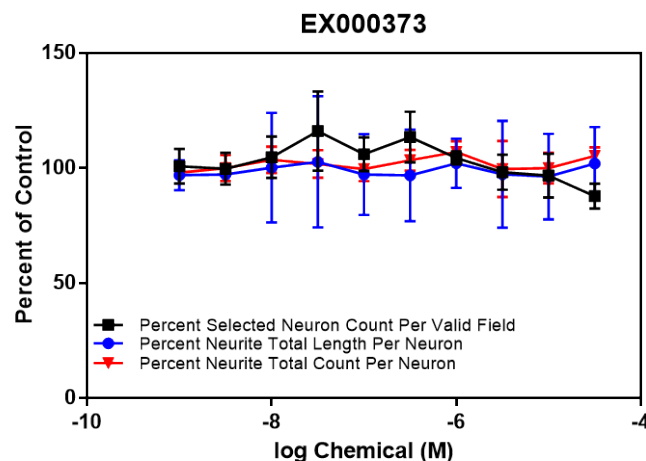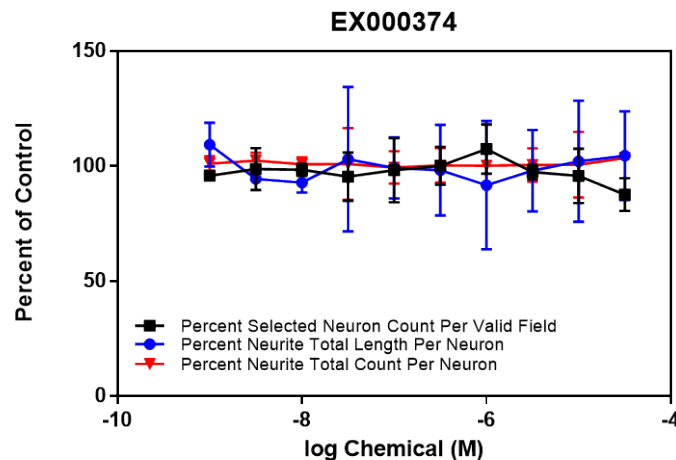Bis1 also decreases network formation.

Robinette et al., 2011

Assay control chemicals perform as expected
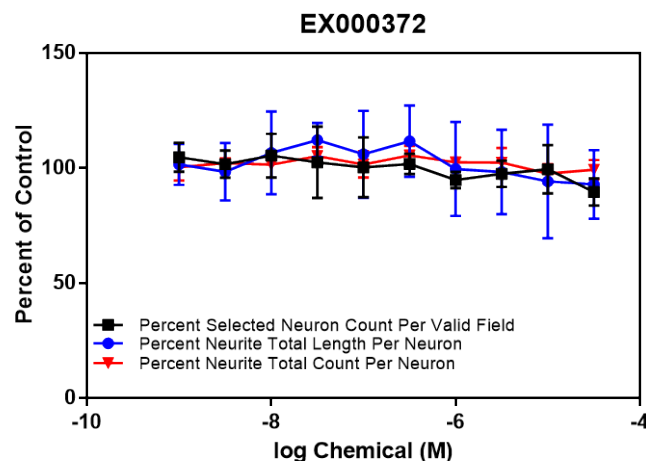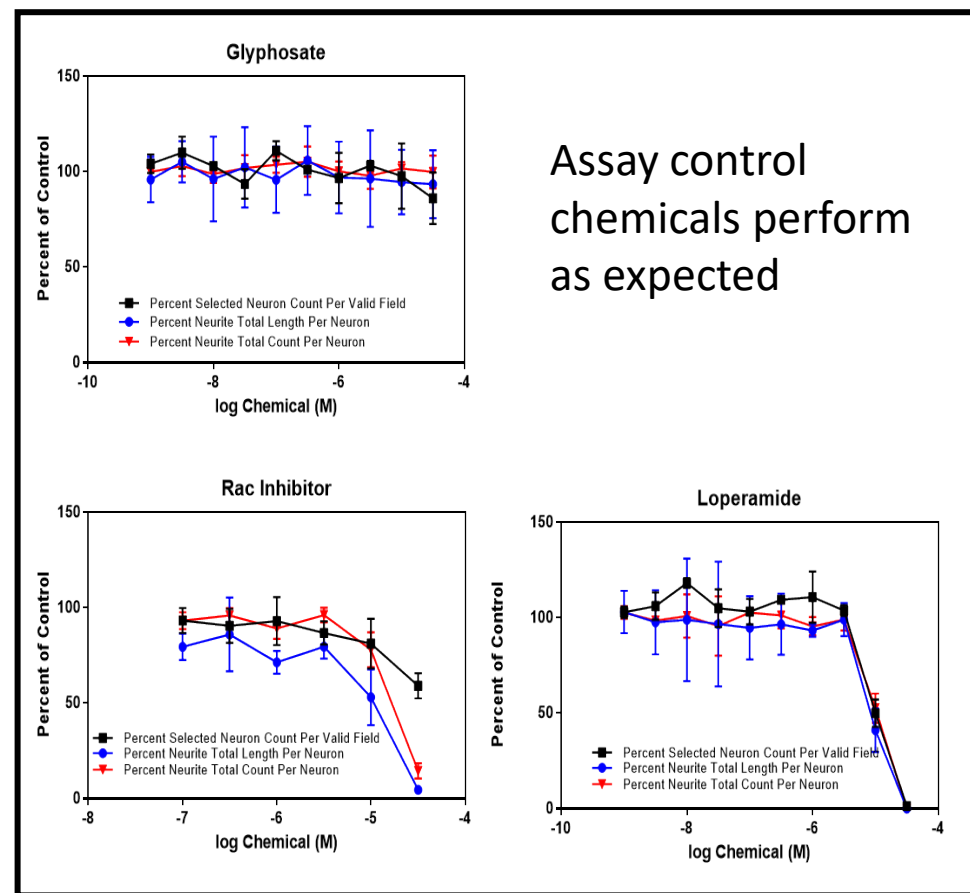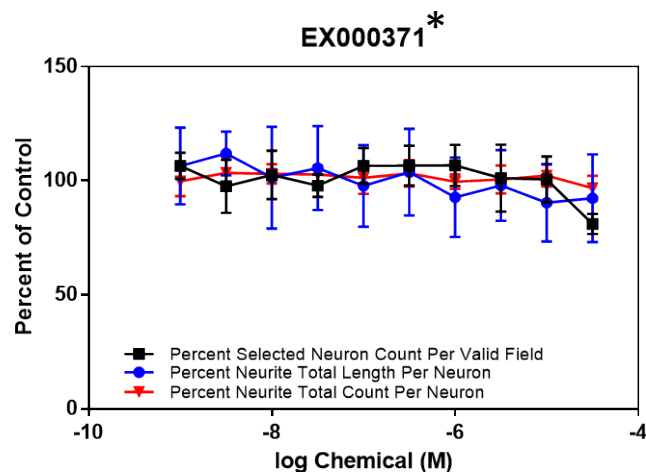
*EX000371 = DL-Glufosinate (GLF)

Conclusion: DL-GLF and analogs have no effects on neurite outgrowth

Assay controls (Glyphosate; negative and Loperamide; positive) produce the expected results
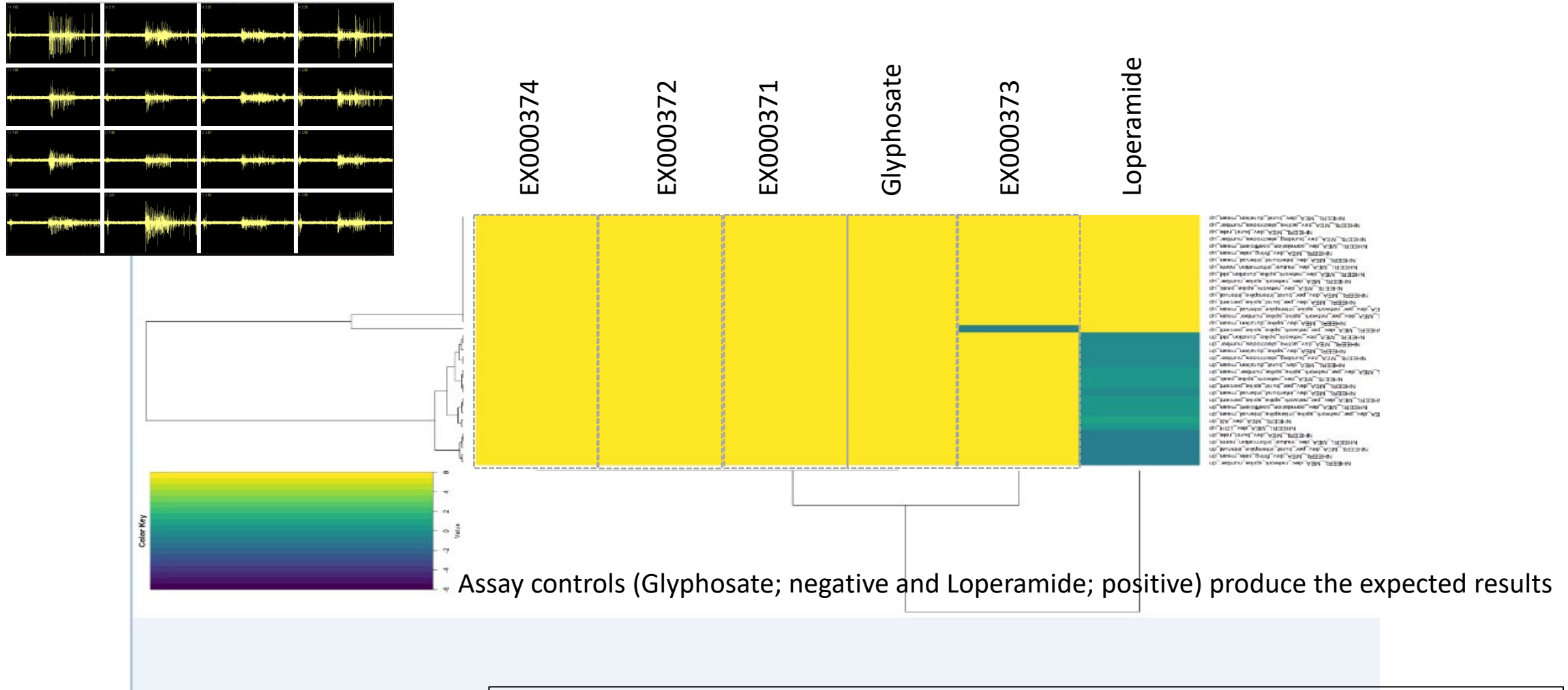
Conclusion: DL-GLF and analogs have no effects on Network Formation

Acute Effects on Network Function
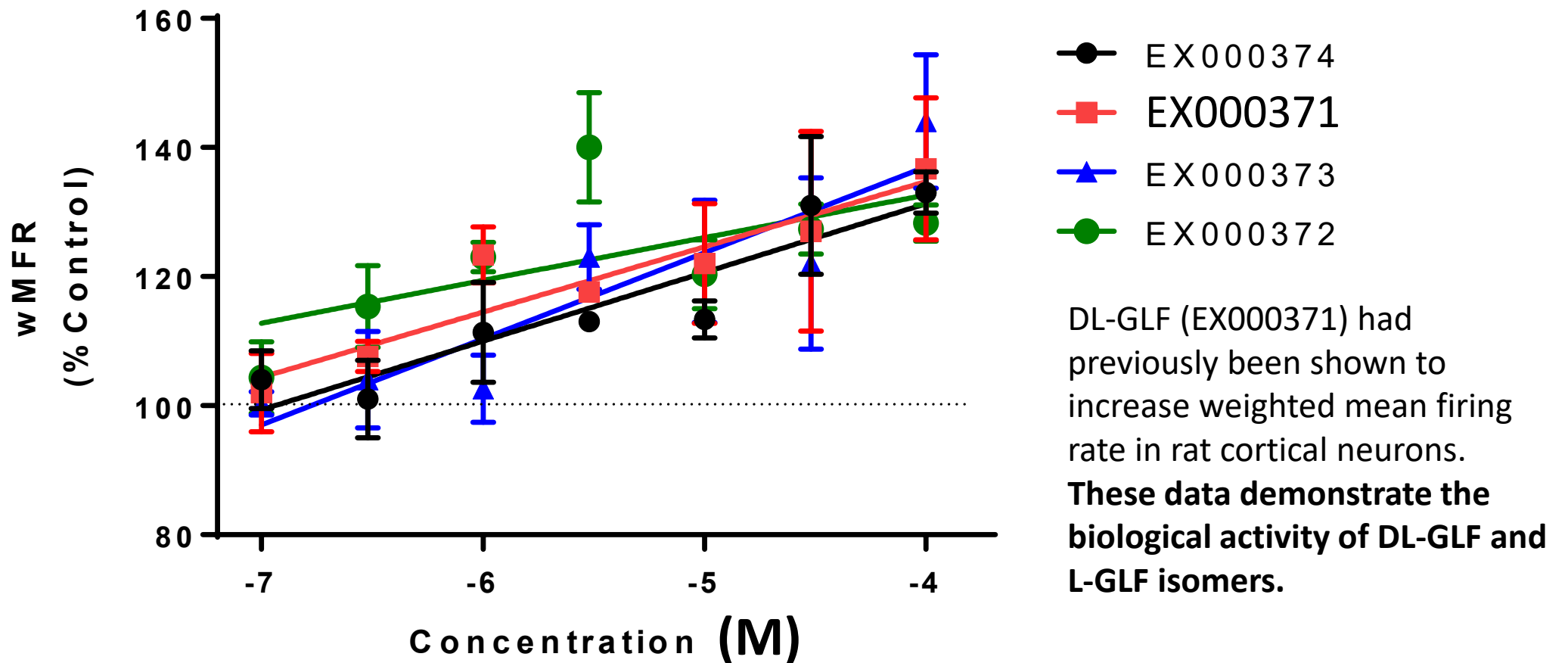
DL-GLF (EX000371) had previously been shown to increase weighted mean firing rate in rat cortical neurons. **These data demonstrate the biological activity of DL-GLF and L-GLF isomers.**

From Guideline study, LOAEL of Compound X = **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 Compound = AED of **45 mg/kg/day** (rats) and 13.5 mg/kg/day (humans)

Summary: At concentrations equivalent to or above the LOAEL from in vivo studies, DL-GLF and analogs did not alter Neurite Outgrowth or Network Formation, but did have acute effects on Network Function